



Goldsborough et al.
USSN 400/25,897
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In the Title:

A marked copy of the title revision has been provided in the attached Appendix I.

Please amend the title to read as follows:

--Methods for the Storage and Synthesis of Nucleic Acids using a Solid Support--

In the Specification:

Marked copies of the specification revisions, but not additions, have been provided in the attached Appendix I.

On page 33 of the application, kindly amend this application to include the attached sequence listing (currently pages 1-4). Please renumber the pages with the claims and the abstract accordingly.

Please replace the paragraph at page 1, lines 5-17, with the following:

-- The present application claims the benefit of U.S. Provisional Application No. 60/175,307, filed January 10, 2000, the disclosure of which is incorporated herein by reference in its entirety.--

Please replace the title of the table at page 27, line 8, with the following:

--**Table 1. Primer Sequences used.** (SEQ ID NOS 1-12, respectively in order of appearance)--

Please replace the paragraph at page 28, line 23, to page 29, line 17, with the following paragraph:

-- The results of the amplification of nucleic acids stored on solid supports are shown in Figures 2-4. Figure 2 shows the results of the amplification of nucleic acids from HeLa cells. Eluted RNA was precipitated from washes taken from 2-mm punches of HeLa cell samples stored at -20° and -70°C for 1 year as described above. The amplification targets were as follows: Panel A; a 626 bp sequence from b-actin mRNA was amplified using the following thermocycling conditions: 94°C for 1 min, followed by 40 cycles of 94°C for 30 s; 60°C for 30 s and 72°C for 1.5 min; forward and reverse primer sequences were 5'CCTCGCCTTTGCCGATCC3' (SEQ ID NO: 9) and 5'GGATCTTCATGAGGTAGTCAGTC3' (SEQ ID NO: 10), respectively. Panel B; a 1.08-kb sequence of RPA (replication protein A) mRNA was amplified using the following thermocycling conditions: 94°C for 1 min, followed by 40 cycles of 94°C for 30 s; 55°C for 30 s and 72°C for 1.5 min; forward and reverse primer sequences were 5'CAAGATGTGGAACAGTGGATTC3' (SEQ ID NO: 7) and 5'CATCTATCTTGATGTTGTAACAAGC3' (SEQ ID NO: 8), respectively. and Panel C: a 5.76-kb sequence of a clathrin-like protein (D21260) mRNA was amplified using the following thermocycling conditions: 94°C for 1 min, followed by 35 cycles of 94°C for 20 s; 60°C for 30 s and 68°C for 7 min; forward and reverse primer sequences were 5'CCCAGTGACAGGAGGAGACCATA3' (SEQ ID NO: 11) and 5'ATCCTGTGCTTTTCTGTGGGAC3' (SEQ ID NO: 12), respectively. For Panels A and B, Lanes 1-3 and 4-6 are from samples stored at -20°C and -70°C, respectively subsequent to sample application onto FTA® GeneCards, whereas lane 7 is a negative control where SUPERScript II RT was

omitted from the RT reaction. Lanes labeled M are a 1 kb ladder size markers. For Panel C, lanes 1, positive control, HeLa RNA, Lanes-2 and 3 are from samples stored at -70°C subsequent to sample application onto FTA® GeneCards, whereas lane 4 is the negative control.--

Please replace the paragraph at page 31, lines 13-29, with the following paragraph:

-- Poly(A+)RNA was directly isolated from 2.25×10^6 BHK-21 cells stored on FTA® paper as described above except that the biotinylated oligonucleotide(dT) had special adapter sequences necessary for library construction. The primer includes a *Not* I recognition site and has the sequence (Biotin)₄ GACTAGTTCTAGAT CGCGAGCGG CCGCCCTTTTT TTTTTTTTTTTT TTTTTTTT (SEQ ID NO: 13); (see WO 98/51699 and United States application serial number 09/076,115). As a positive control, poly(A+) RNA was isolated total RNA prepared by TRIzol reagent from the same number of cells. Double-stranded cDNA was made and cloned into plasmid vectors as described in WO 98/51699 and United States application serial number 09/076,115. The number of primary clones obtained from the poly(A+)RNA was the same whether the mRNA was isolated directly from FTA® or from TRIzol-purified total RNA. The average insert size of the libraries was determined by colony PCR using primers to the plasmid vector. The average insert size for the FTA®-derived material was greater than that for the library constructed from the positive control poly(A+)RNA, 1000bp vs 600 bp. This indicates that cDNA libraries of good quality can be made from mRNA isolated directly from samples stored on FTA®.--

On page 19 of the application, kindly insert the following text after line 18:

--In pertinent part, United States patent application serial number 09/076,115 reads as follows:

SUMMARY OF THE INVENTION

The present invention is directed to methods useful for the production and isolation of nucleic acid molecules (single- and double-stranded) from small amounts of input nucleic acid molecules. More particularly, the invention provides methods for the production of a cDNA molecule (single- or double-stranded) from an RNA template (*e.g.*, single-stranded mRNA or polyA⁺ RNA) by using ligand-coupled primer-adaptor molecules. Such primer-adaptor molecules may also be used in accordance with the invention to isolate mRNA or polyA⁺ RNA molecules from an RNA-containing sample.

Specifically, the invention is directed to a method for producing a nucleic acid molecule comprising mixing a nucleic acid template, preferably a mRNA or a polyA⁺ RNA molecule, with a polypeptide having polymerase and/or reverse transcriptase activity and a primer-adaptor nucleic acid molecule, wherein the primer-adaptor nucleic acid molecule comprises one or more ligand molecules and one or more cleavage sites (preferably a restriction endonuclease cleavage site or an endonuclease cleavage site). This primer-adaptor may be designed to hybridize to any portion of the template. Upon incubation under appropriate conditions, a first nucleic acid molecule (*e.g.*, a single-stranded cDNA) complementary to all or a portion of the template is made. This first nucleic acid molecule contains the primer-adaptor (preferably at or near its termini) which facilitates isolation of the first nucleic acid molecule and/or any nucleic acid

molecule hybridized to the first nucleic acid molecule. Thus, if the first nucleic acid molecule (*e.g.*, single-stranded cDNA) serves as a template to make a second nucleic acid molecule (*e.g.*, forming a double-stranded molecule such as a double-stranded cDNA), the double-stranded molecule can be isolated using the primer-adaptor contained in the molecule. Likewise, the template-first nucleic acid hybrid formed during synthesis of the first nucleic acid molecule can be isolated. If desired, the primer-adaptor may be included at any step or at multiple steps during nucleic acid synthesis. For example, primer-adaptor molecules may be added during the first, second, third, fourth, etc., synthesis step (the first synthesis step making a nucleic acid molecule complementary to all or a portion of the template) or can be added in multiple or all such synthesis steps. Multiple synthesis with primer-adaptor molecules may result in a synthesized nucleic acid molecule having more than one primer-adaptor.

To isolate mRNA or polyA⁺ RNA from RNA-containing samples, one or more mRNA- or polyA⁺ RNA-specific primer-adaptors is used. Such a primer-adaptor is hybridized to the mRNA and/or polyA⁺ RNA to form a primer-adaptor/polyA⁺ RNA hybrid. The primer-adaptor can then facilitate isolation of the mRNA and/or polyA⁺ RNA from a sample. In this aspect of the invention, since the primer-adaptor is hybridized to the molecule of interest and can be removed by denaturation, cleavage sites in the primer-adaptor are not needed.

The primer-adaptor molecules of the invention may also be used to isolate specific nucleic acid sequences. By using one or more target-specific primer-adaptors capable of hybridizing to one or more sequences of interest, the invention allows selection and isolation of specific nucleic acid molecules (*e.g.*, genes or portions thereof) from a population of nucleic acid molecules. In accordance with the invention, the use of two or more such target-specific primer-adaptors (each directed to a different sequence) allows selection of more than one different sequence of interest. Alternatively, two or more target-specific primer-

adapters directed to different portions of a sequence of interest facilitates selection of such sequences by reducing background contamination. Because, in this aspect of the invention, the target-specific primer-adapter hybridizes to the desired molecule and can be removed by denaturation, cleavage sites in the target-specific primer-adapter are not needed.

In accordance with the invention, the primer-adapter molecules facilitate isolation of molecules comprising such primer-adapters by relying on the ligand portion of the primer-adapter. After the primer-adapter is bound (hybridized or incorporated during synthesis) to the nucleic acid molecule, the ligand portion of the primer-adapter allows selective isolation of the molecule containing the primer-adapter. Such isolation may be accomplished by ligand-hapten interactions, where the hapten is bound to, for example, a solid support. Once bound to the solid support, the molecules of interest (primer-adapter containing nucleic acid molecules) can be separated from contaminating nucleic acids and proteins by washing the support matrix with a solution, preferably a buffer or water. Cleavage of one or more of the cleavage sites within the primer-adapter then allows for removal of the nucleic acid molecule of interest from the solid support, leaving the ligand bound to the hapten of the solid support. Alternatively, where the primer-adapter is hybridized to the nucleic acid molecule of interest, isolation can be accomplished by denaturation of the primer-adapter from the desired molecules and/or by cleavage of the cleavage sites within the primer-adapter molecule.

Preferred solid supports for use in the invention include, but are not limited to, nitrocellulose, diazocellulose, glass, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, Sepharose, agar, starch, nylon, latex beads, magnetic beads, paramagnetic beads, superparamagnetic beads or microtitre plates and most preferably a magnetic bead, a paramagnetic bead or a

superparamagnetic bead, that comprises one or more hapten molecules specifically recognizing and binding to the ligand molecule.

Particularly preferred hapten molecules according to this aspect of the invention include without limitation: (i) avidin and streptavidin; (ii) protein A, protein G, a cell-surface Fc receptor or an antibody-specific antigen; (iii) an enzyme-specific substrate; (iv) polymyxin B or endotoxin-neutralizing protein (ENP); (v) Fe^{+++} ; (vi) a transferrin receptor; (vii) an insulin receptor; (viii) a cytokine (*e.g.*, growth factor, interleukin or colony-stimulating factor) receptor; (ix) CD4; (x) spectrin or fodrin; (xi) ICAM-1 or ICAM-2; (xii) C3bi, fibrinogen or Factor X; (xiii) ankyrin; (xiv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xvi) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xvii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xviii) vitronectin; (xix) fibronectin; (xx) collagen; (xxi) laminin; (xxii) glycophorin; (xxiii) Mac-1; (xxiv) LFA-1; (xxv) β -actin; (xxvi) gp120; (xxvii) cytokines (growth factors, interleukins or colony-stimulating factors); (xxviii) insulin; (xxix) ferrotransferrin; (xxx) apotransferrin; (xxxi) lipopolysaccharide; (xxxii) an enzyme; (xxxiii) an antibody; and (xxxiv) biotin.

Particularly preferred ligand molecules for use according to the invention, which correspond in order to the above-described hapten molecules, include without limitation: (i) biotin; (ii) an antibody; (iii) an enzyme; (iv) lipopolysaccharide; (v) apotransferrin; (vi) ferrotransferrin; (vii) insulin; (viii) cytokines (growth factors, interleukins or colony-stimulating factors); (ix) gp120; (x) β -actin; (xi) LFA-1; (xii) Mac-1; (xiii) glycophorin; (xiv) laminin; (xv) collagen; (xvi) fibronectin; (xvii) vitronectin; (xviii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xix) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xx) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xxi) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xxii) ankyrin; (xxiii) C3bi, fibrinogen or Factor X; (xxiv) ICAM-1 or ICAM-2; (xxv) spectrin or fodrin; (xxvi) CD4; (xxvii) a cytokine (*e.g.*, growth factor, interleukin

or colony-stimulating factor) receptor; (xxviii) an insulin receptor; (xxix) a transferrin receptor; (xxx) Fe^{+++} ; (xxxi) polymyxin B or endotoxin-neutralizing protein (ENP); (xxxii) an enzyme-specific substrate; (xxxiii) protein A, protein G, a cell-surface Fc receptor or an antibody-specific antigen; and (xxxiv) avidin and streptavidin.

The invention thus relates to a method for making a nucleic acid molecule comprising

(a) mixing a polypeptide having polymerase and/or reverse transcriptase activity with a nucleic acid template and a primer-adapter of the invention; and

(b) incubating the mixture under conditions sufficient to make a first nucleic acid molecule which comprises the primer-adapter (preferably at or near its 5' or 3' termini) and which is complementary to all or a portion of the template. [If a DNA polymerase is used in accordance with the invention, the primer-adapter may be located at or near the 3' terminus, while if a reverse transcriptase is used the primer-adapter may be located at or near the 5' terminus of the synthesized nucleic acid molecule. In accordance with the invention, the first nucleic acid molecule may be used as a template to make a second nucleic acid molecule complementary to all or a portion of the first nucleic acid molecule. If a primer-adapter is used in this synthesis, a double-stranded nucleic acid molecule is produced which comprises a primer-adapter at or near each terminus, although on different strands of the molecule. However, the primer-adapter may be omitted from this second synthesis thereby providing for a double-stranded nucleic acid molecule having a primer-adapter at one terminus.

If desired, the primer-adapters of the invention may be used in methods for amplifying a nucleic acid molecule. Such methods comprise

(a) contacting a polypeptide having polymerase and/or reverse transcriptase activity with a nucleic acid template and two or more primer-adapters; and

(b) incubating the mixture under conditions sufficient to amplify a nucleic acid molecule complementary to all or a portion of the template.

Such amplification methods may specifically comprise

(a) contacting a double-stranded nucleic acid molecule to be amplified with a polypeptide having polymerase and/or reverse transcriptase activity, a first primer-adapter complementary to a portion of the first strand of the double-stranded molecule and a second primer-adapter complementary to a portion of the second strand of the double-stranded molecule;

(b) incubating the mixture under conditions sufficient to make a third strand nucleic acid molecule comprising the first primer-adapter and which is complementary to all or a portion of the first strand, and a fourth strand nucleic acid molecule comprising the second primer-adapter and which is complementary to all or a portion of the second strand;

(c) denaturing the second and fourth, and the first and third, strands to form single-stranded nucleic acid molecules; and

(d) repeating steps (a)-(c) one or more times.

In this aspect of the invention, the first primer-adapter or the second primer-adapter may be replaced with any oligonucleotide primer to prime synthesis of a nucleic acid molecule.

In a preferred aspect of the invention, RNA (*e.g.*, mRNA or polyA⁺ RNA) is used as a template for DNA synthesis. This preferred method comprises mixing the RNA template with one or more polypeptides having reverse transcriptase activity and a primer and incubating the mixture under conditions sufficient to make a DNA (*e.g.*, a cDNA) molecule complementary to all or a portion of the RNA template. The synthesized DNA molecule may then be used

as a template for additional DNA synthesis or DNA amplification. In accordance with this aspect of the invention, a cDNA library may be produced when using a population of RNA molecules (for example, RNA isolated from a cell or tissue).

For isolating mRNA or polyA⁺ RNA in accordance with the invention, the method may specifically comprise:

- (a) obtaining a sample containing (or thought to contain) mRNA and/or polyA⁺ RNA;
- (b) contacting the sample with one or more primer-adapters capable of selectively binding to mRNA and/or polyA⁺ RNA; and
- (c) isolating the mRNA and/or polyA⁺ RNA from the sample.

For isolating specific or desired nucleic acid molecules, the invention may specifically comprise:

- (a) obtaining a sample containing (or thought to contain) one or more desired nucleic acid molecules;
- (b) contacting the sample with one or more primer-adapters capable of selectively binding to one or more of the desired nucleic acid molecules; and
- (c) isolating the desired nucleic acid molecules from the sample.

In a preferred aspect, the sample containing the desired molecules is a population of double-stranded or single-stranded cDNA molecules. Thus, the invention relates to a method of isolating one or more desired nucleic acid molecules comprising:

- (a) obtaining a sample containing a population of cDNA molecules which contain (or are thought to contain) one or more desired cDNA molecules;
- (b) contacting the sample with one or more target-specific primer-adapters capable of specifically binding to one or more of the desired cDNA molecules; and
- (c) isolating the desired cDNA molecules from the sample.

In accordance with the invention, the target-specific primer-adapters may be used in selection of a specific cDNA molecule after the cDNA molecule is synthesized from the RNA template (binding to the RNA/cDNA double-stranded molecule or binding to the single-stranded cDNA molecule after removing the RNA strand). Alternatively, the target-specific primer-adapters may be used to bind the double-stranded cDNA molecule. Such target-specific primer-adapters may also be used in accordance with the invention to select one or more desired molecules from a population of amplified nucleic acid molecules.

The invention is also directed to vectors, including expression vectors, comprising the cDNA molecules or nucleic acid molecules produced in accordance with the invention, and to host cells comprising these cDNA molecules, nucleic acid molecules or vectors. The invention also provides methods for producing a recombinant polypeptide comprising culturing these host cells under conditions favoring the expression of a recombinant polypeptide and isolating the polypeptide, and provides recombinant polypeptides produced according to these methods.

In other preferred aspects, the invention is directed to kits for the production of a nucleic acid molecule or a cDNA molecule comprising a carrier means such as a box, carton, or the like being compartmentalized to receive in close confinement therein one or more containers, such as tubes, vials, bottles, ampules and the like, wherein a first container comprises a primer-adaptor molecule comprising one or more ligand molecules, preferably biotin, and which comprises one or more cleavage sites, preferably one or more restriction endonuclease cleavage sites. The invention is also directed to such kits comprising additional containers which may contain one or more polypeptides having reverse transcriptase activity and/or polymerase activity. According to the invention, more than one polypeptide may be included in the same or different containers. The invention is also directed to kits comprising additional containers

which may contain a solid support having one or more haptens capable of specifically binding the ligand or ligands of the primer-adapters of the invention. The invention is also directed to kits comprising additional containers which may contain one or more endonucleases which recognize and cleave the cleavage sites in the primer-adapters of the invention.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is particularly suited for the rapid production and isolation of cDNA libraries from small amounts of poly A⁺ RNA or mRNA in a high-throughout manner. In a preferred aspect of the invention, a population of single-stranded poly A⁺ RNA or mRNA is hybridized in solution with a ligand-coupled primer adapter (non-specific or gene-specific). As used herein, the term "primer-adapter" refers to a nucleic acid molecule which is capable of specifically binding (*e.g.*, hybridizing) to a template nucleic acid molecule (*e.g.*, a mRNA or polyA⁺ RNA molecule). In a particularly preferred embodiment of the invention, the primer-adapter allows priming of the transcription, reverse transcription, polymerization or elongation of a nucleic acid molecule complementary to all or a portion of the template nucleic acid molecule.

According to the invention, the first and second strand cDNA reactions are preferably performed in one tube, introducing the ligand at or near the 3' end of the double-stranded cDNA produced. The ligand-coupled cDNA may then be isolated by binding to a solid support coupled with a hapten to which the cDNA will bind through ligand-hapten interactions, thereby allowing the concentration of the cDNA and exchange of the buffer without organic extraction and

precipitation. Subsequently, the bound cDNA is released from the solid phase support by restriction enzyme digestion. This asymmetric cDNA is then cloned directionally into a vector that contains the appropriate termini (one terminus matches the restriction site used to release the cDNA and the other terminus is blunt ended). Subsequent or prior to cloning into a vector, specific cDNA sequences (*e.g.*, genes or gene fragments) may be selectively isolated using target-specific primer-adapters of the invention. In addition to the elimination of multiple time-consuming extractions and precipitations, the methods of the invention eliminate the need for DNA adapters and cDNA fractionation (normally a necessary step to remove excess unligated adapters). The invention thus facilitates rapid production and isolation of larger amounts of cDNA and the construction of cDNA libraries from nanogram amounts of poly A⁺ RNA or mRNA without the need for PCR amplification. The invention also provides a simple selection technique which allows isolation of desired genes or gene fragments from the constructed cDNA library.

Sources of Nucleic Acid Template Molecules

Using the methods of the invention, nucleic acid molecules and in particular cDNA molecules may be prepared from a variety of nucleic acid template molecules. Preferred nucleic acid molecules for use in the present invention include single-stranded or double-stranded RNA. More preferred nucleic acid molecules include polyadenylated RNA (polyA⁺ RNA), messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA) molecules, and most preferred are mRNA and polyA⁺ RNA molecules.

The nucleic acid template molecules that are used to prepare nucleic acid or cDNA molecules according to the methods of the present invention may be prepared synthetically according to standard organic chemical synthesis methods that will be familiar to one of ordinary skill. More preferably, the nucleic acid

template molecules may be obtained from natural sources, such as a variety of cells, tissues, organs or organisms. Cells that may be used as sources of nucleic acid molecules may be prokaryotic (bacterial cells, including those of species of the genera *Escherichia*, *Bacillus*, *Serratia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Chlamydia*, *Neisseria*, *Treponema*, *Mycoplasma*, *Borrelia*, *Legionella*, *Pseudomonas*, *Mycobacterium*, *Helicobacter*, *Erwinia*, *Agrobacterium*, *Rhizobium*, and *Streptomyces*) or eukaryotic (including fungi (especially yeasts), plants, protozoans and other parasites, and animals including insects (particularly *Drosophila* spp. cells), nematodes (particularly *Caenorhabditis elegans* cells), and mammals (particularly human cells)).

Mammalian somatic cells that may be used as sources of nucleic acids include blood cells (reticulocytes and leukocytes), endothelial cells, epithelial cells, neuronal cells (from the central or peripheral nervous systems), muscle cells (including myocytes and myoblasts from skeletal, smooth or cardiac muscle), connective tissue cells (including fibroblasts, adipocytes, chondrocytes, chondroblasts, osteocytes and osteoblasts) and other stromal cells (e.g., macrophages, dendritic cells, Schwann cells). Mammalian germ cells (spermatocytes and oocytes) may also be used as sources of nucleic acids for use in the invention, as may the progenitors, precursors and stem cells that give rise to the above somatic and germ cells. Also suitable for use as nucleic acid sources are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus.

Any of the above prokaryotic or eukaryotic cells, tissues and organs may be normal, diseased, transformed, established, progenitors, precursors, fetal or embryonic. Diseased cells may, for example, include those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS) or

parasites), in genetic or biochemical pathologies (*e.g.*, cystic fibrosis, hemophilia, Alzheimer's disease, muscular dystrophy or multiple sclerosis) or in cancerous processes. Transformed or established animal cell lines may include, for example, COS cells, CHO cells, VERO cells, BHK cells, HeLa cells, HepG2 cells, K562 cells, F9 cells and the like. Other cells, cell lines, tissues, organs and organisms suitable as sources of nucleic acids for use in the present invention will be apparent to one of ordinary skill in the art.

Once the starting cells, tissues, organs or other samples are obtained, nucleic acid molecules (such as mRNA) may be isolated therefrom by methods that are well-known in the art (*See, e.g.*, Maniatis, T., *et al.*, *Cell* 15:687-701 (1978); Okayama, H., and Berg, P., *Mol. Cell. Biol.* 2:161-170 (1982); Gubler, U., and Hoffman, B.J., *Gene* 25:263-269 (1983)). As discussed, the invention provides an improvement in isolating mRNA and/or polyA⁺ RNA from samples. The use of the primer-adapters of the invention, which specifically recognize and bind polyA⁺ RNA or mRNA, allows for such selection. Preferably, the primer-adaptor recognizes and hybridizes to the polyA tail of the mRNA or polyA⁺ RNA. Such primer-adapters may include an primer-adapters comprising oligo(dT). Once bound, use of the ligand portion of the primer-adaptor allows isolation of the desired RNA molecule. The polyA⁺ RNA or mRNA molecules thus isolated may then be used to prepare cDNA molecules and cDNA libraries using the methods of the present invention.

Synthesis of Nucleic Acid Molecules

In the practice of the invention, nucleic acid molecules and in particular cDNA molecules or cDNA libraries comprising one or more ligand molecules are produced by mixing a nucleic acid template obtained as described above, which is preferably a mRNA molecule or a polyA⁺ RNA molecule, with one or more polypeptides having polymerase activity and/or reverse transcriptase activity and

with a one or more primer-adapters of the invention. Under conditions favoring the reverse transcription and/or polymerization of the input nucleic acid molecule, synthesis of a nucleic acid molecule complementary to all or a portion of the template is accomplished. Preferred polypeptides (*e.g.*, enzymes) having reverse transcriptase and/or polymerase activity to be used in the present invention include, but are not limited to, Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase, Human Immunodeficiency Virus (HIV) reverse transcriptase, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, *Thermus thermophilus* (*Tth*) DNA polymerase, *Thermus aquaticus* (*Taq*) DNA polymerase, *Thermotoga neopolitana* (*Tne*) DNA polymerase, *Thermotoga maritima* (*Tma*) DNA polymerase, *Thermococcus litoralis* (*Tli* or VENT) DNA polymerase, *Pyrococcus furiosus* (*Pfu* or DEEPVENT) DNA polymerase, *Pyrococcus woosii* (*Pwo*) DNA polymerase, *Bacillus sterothermophilus* (*Bst*) DNA polymerase, *Sulfolobus acidocaldarius* (*Sac*) DNA polymerase, *Thermoplasma acidophilum* (*Tac*) DNA polymerase, *Thermus flavus* (*Tfl/Tub*) DNA polymerase, *Thermus ruber* (*Tru*) DNA polymerase, *Thermus brockianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicum* (*Mth*) DNA polymerase, and mutants, variants and derivatives thereof. Particularly preferred for use in the invention are the variants of these enzymes that are substantially reduced in RNase H activity. By an enzyme "substantially reduced in RNase H activity" is meant that the enzyme has less than about 20%, more preferably less than about 15%, 10% or 5%, and most preferably less than about 2%, of the RNase H activity of a wildtype or "RNase H⁺" enzyme such as wildtype M-MLV or AMV reverse

transcriptases. The RNase H activity of any enzyme may be determined by a variety of assays, such as those described, for example, in U.S. Patent No. 5,244,797, in Kotewicz, M.L., *et al.*, *Nucl. Acids Res.* 16:265 (1988) and in Gerard, G.F., *et al.*, *FOCUS* 14(5):91 (1992), the disclosures of all of which are fully incorporated herein by reference.

Any ligand to which a hapten molecule will bind may be used to form the ligand-coupled primer-adapter molecule used in the present methods. Suitable ligands for this purpose include, but are not limited to: (i) biotin; (ii) an antibody; (iii) an enzyme; (iv) lipopolysaccharide; (v) apotransferrin; (vi) ferrotransferrin; (vii) insulin; (viii) cytokines (growth factors, interleukins or colony-stimulating factors); (ix) gp120; (x) β -actin; (xi) LFA-1; (xii) Mac-1; (xiii) glycophorin; (xiv) laminin; (xv) collagen; (xvi) fibronectin; (xvii) vitronectin; (xviii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xix) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIB}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xx) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xxi) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xxii) ankyrin; (xxiii) C3bi, fibrinogen or Factor X; (xxiv) ICAM-1 or ICAM-2; (xxv) spectrin or fodrin; (xxvi) CD4; (xxvii) a cytokine (*e.g.*, growth factor, interleukin or colony-stimulating factor) receptor; (xxviii) an insulin receptor; (xxix) a transferrin receptor; (xxx) Fe^{+++} ; (xxxi) polymyxin B or endotoxin-neutralizing protein (ENP); (xxxii) an enzyme-specific substrate; (xxxiii) protein A, protein G, a cell-surface Fc receptor or an antibody-specific antigen; and (xxxiv) avidin and streptavidin. Most preferred for use in the methods of the invention is biotin. The ligand-coupled primer-adapter nucleic acid molecules, in which one or more ligand molecules are attached (preferably covalently) to one or more nucleotides of the primer-adapter molecule * * * may be produced using conventional organic synthesis methods that are familiar to one of ordinary skill in the art. For example, the oligonucleotide may be biotinylated at the 5' terminus by first producing 5' amino (NH_2) groups followed by Cab-NHS ester addition (Langer, P.R., *et al.*, *Proc. Natl. Acad. Sci. USA* 78:6633 (1981)).

In a particularly preferred aspect of the invention, a primer-adapter molecule comprising one or more, two or more, three or more or four or more ligand molecules, most preferably biotin molecules, is prepared.

In addition to the ligand molecules, the primer-adapter molecule also preferably comprises one or more endonuclease cleavage sites, preferably restriction endonuclease cleavage sites. These sites facilitate the release of the newly synthesized nucleic acid molecule comprising the primer-adapter from the hapten-coupled solid support. Examples of endonucleases which can be used in accordance with the invention include, but are not limited to, GeneII. Examples of restriction endonucleases which can be used in accordance with the invention include, but are not limited to, *AluI*, *Eco47 III*, *EcoRV*, *FspI*, *HpaI*, *MscI*, *NruI*, *PvuII*, *RsaI*, *ScaI*, *SmaI*, *SspI*, *StuI*, *ThaI*, *AvaI*, *BamHI*, *BanII*, *BglII*, *ClaI*, *EcoRI*, *HindIII*, *HpaII*, *KpnI*, *MseI*, *NcoI*, *NdeI*, *NotI*, *PstI*, *PvuI*, *SacI/SstI*, *SalI*, *XbaI*, *XhoI* and *I-CeuI*.

The restriction endonuclease sites engineered into the primer-adapter molecule are preferably chosen to result in either blunt ends or sticky ends. Examples of blunt-end restriction enzymes, the recognition sites for which may be engineered into the primer-adapter molecules of the invention, include without limitation *AluI*, *Eco47 III*, *EcoRV*, *FspI*, *HpaI*, *MscI*, *NruI*, *PvuII*, *RsaI*, *ScaI*, *SmaI*, *SspI*, *StuI* and *ThaI*.

Examples of sticky-end restriction enzymes, the recognition sites for which may be engineered into the primer-adapter molecules of the invention, include without limitation *AvaI*, *BamHI*, *BanII*, *BglII*, *ClaI*, *EcoRI*, *HindIII*, *HpaII*, *KpnI*, *MseI*, *NcoI*, *NdeI*, *NotI*, *PstI*, *PvuI*, *SacI/SstI*, *SalI*, *XbaI*, *XhoI* and *I-CeuI*.

In a particularly preferred aspect of the invention, the primer-adapter molecule is engineered to contain a site recognized by rare cutting restriction endonucleases, for example, those recognizing 8 or more bases (*e.g.*, a 8- basepair

cutter, etc.). Such restriction sites may include a *NotI* restriction site, a *I-CeuI* restriction site, a *PI-PspI* restriction site, an *I-PpoI* restriction site, a *PI-TliI* restriction site and a *PI-FceI* restriction site. The above-mentioned restriction enzymes, and others that may be equivalently used in the methods of the present invention, are available commercially, for example from Life Technologies, Inc. (Rockville, MD). See also Roberts, R.J., *Nucl. Acids Res.* 17(Suppl.):r347-r387 (1989), for other examples of restriction enzymes and their cleavage sites.

Once the ligand-coupled primer-adaptor molecule has been obtained, it is used to produce nucleic acid molecules from the input nucleic acid using any of a number of well-known techniques. Such synthetic techniques involve hybridization of the primer-adaptor to the nucleic acid template and extending the primer-adaptor to make a nucleic acid molecule complementary to all or a portion of the template. Such synthesis is accomplished in the presence of nucleotides (*e.g.*, deoxyribonucleoside triphosphates (dNTPs), dideoxyribonucleoside triphosphates (ddNTPs) or derivatives thereof) and one or more polypeptides having polymerase and/or reverse transcriptase activity. The primer-adaptors of the invention may be used in any nucleic acid synthesis reaction including cDNA synthesis, nucleic acid amplification and nucleic acid sequencing, using well-known techniques. For synthesis of cDNA, the primer-adaptor molecules of the invention may be used in conjunction with methods of cDNA synthesis such as those described in Example 1 below, or others that are well-known in the art (*see, e.g.*, Gubler, U., and Hoffman, B.J., *Gene* 25:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol.* 152:316-325 (1987); Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1987)), to produce cDNA molecules or libraries.

Alternatively, the primer adaptor molecules of the invention may be used in single-tube synthesis of cDNA molecules according to the invention. In this

approach, the input nucleic acid molecule (preferably a mRNA or polyA⁺ RNA molecule) is hybridized in solution with the primer-adaptor molecule of the invention, and the hybridized complex is contacted with a polypeptide (*e.g.*, an enzyme) having reverse transcriptase activity (which is preferably any of those described above) in the presence of dNTPs and cofactors needed for cDNA synthesis. Following first strand synthesis, the second cDNA strand may then be synthesized in the same reaction vessel by a modified Gubler-Hoffman reaction (D'Alessio, J.M., *et al.*, *Focus* 9:1 (1987)). Other techniques of cDNA synthesis in which the methods of the invention may be advantageously used will be readily apparent to one of ordinary skill in the art.

Isolation of Nucleic Acid Molecules

According to the present methods, single-stranded or double-stranded nucleic acid molecules (*e.g.*, cDNA molecules or cDNA libraries) comprising one or more primer-adapters will be produced. Such nucleic acid molecules or libraries may then be rapidly isolated from solution by binding the nucleic acid molecules to a solid support comprising one or more hapten molecules that will bind the ligands.

In the practice of the invention, any solid support to which a ligand-specific hapten molecule can be bound may be used. Preferred such solid phase supports include, but are not limited to, nitrocellulose, diazocellulose, glass, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, Sepharose, agar, starch, nylon, beads and microtitre plates. Preferred are beads made of glass, latex or a magnetic material, and particularly preferred are magnetic, paramagnetic or superparamagnetic beads. Linkage of the hapten molecule to the solid support can be accomplished by any method of hapten coupling such as covalent, hydrophobic or ionic coupling (including coating) that will be familiar to one of ordinary skill in the art.

According to the invention, any hapten molecule having the capability of binding the ligand molecule that is coupled to the primer-adaptor molecule (and that therefore is contained in the nucleic acid molecules produced by the present methods) may be used. Particularly preferred hapten molecules for use in the invention (which correspond in order to the ligand molecules listed above) include without limitation: (i) avidin and streptavidin; (ii) protein A, protein G, a cell-surface Fc receptor or an antibody-specific antigen; (iii) an enzyme-specific substrate; (iv) polymyxin B or endotoxin-neutralizing protein (ENP); (v) Fe^{+++} ; (vi) a transferrin receptor; (vii) an insulin receptor; (viii) a cytokine (*e.g.*, growth factor, interleukin or colony-stimulating factor) receptor; (ix) CD4; (x) spectrin or fodrin; (xi) ICAM-1 or ICAM-2; (xii) C3bi, fibrinogen or Factor X; (xiii) ankyrin; (xiv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xvi) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIB}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xvii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xviii) vitronectin; (xix) fibronectin; (xx) collagen; (xxi) laminin; (xxii) glycophorin; (xxiii) Mac-1; (xxiv) LFA-1; (xxv) β -actin; (xxvi) gp120; (xxvii) cytokines (growth factors, interleukins or colony-stimulating factors); (xxviii) insulin; (xxix) ferrotransferrin; (xxx) apotransferrin; (xxxii) lipopolysaccharide; (xxxii) an enzyme; (xxxiii) an antibody; and (xxxiv) biotin.

For example, in a preferred aspect of the invention where the primer-adaptor molecule and the newly synthesized nucleic acid molecules comprise biotin, a biotin-binding hapten such as avidin or streptavidin may be linked to the solid support. In a particularly preferred such aspect, the solid support used is avidin- or streptavidin-coupled magnetic, paramagnetic or superparamagnetic beads which are commercially available, for example, from Dynal A.S. (Oslo, Norway) or from Sigma (St. Louis, Missouri). Of course, the choice of hapten will depend upon the choice of ligand used in the production of the primer-adaptor molecule; appropriate haptens for use in the methods of the invention will thus be familiar to one of ordinary skill in the art.

To isolate the nucleic acid molecules produced by the methods of the invention, the solution comprising the nucleic acid molecules which comprise the primer-adapters of the invention is contacted with the hapten-coupled solid support under conditions favoring binding of the ligand by the hapten. Typically, these conditions include incubation in a buffered salt solution, preferably a TRIS-, phosphate-, HEPES- or carbonate-buffered sodium chloride solution, more preferably a TRIS-buffered sodium chloride solution, still more preferably a solution comprising about 10-100 mM TRIS-HCl and about 300-2000 mM NaCl, and most preferably a solution comprising about 10 mM TRIS-HCl and about 1 M NaCl, at a pH of about 6-9, more preferably a pH of about 7-8, still more preferably a pH of about 7.2-7.6, and most preferably a pH of about 7.5. Incubation is preferably conducted at 0°C to about 25°C, and most preferably at about 25°C, for about 30-120 minutes, preferably about 45-90 minutes, and most preferably about 60 minutes, to allow the binding of the ligand-coupled nucleic acid molecules to the hapten-coupled solid support.

Once the nucleic acid molecules have been bound to the solid phase support, unwanted or contaminant materials (such as buffers and enzymes from first and second strand synthesis reactions, untranscribed input RNA molecules, etc.) may be eliminated by simply removing them in the supernatants. For example, in a preferred aspect in which biotinylated cDNA molecules are bound to a avidin- or streptavidin-coupled solid phase, the contaminants may be removed by gently aspirating and discarding the supernatants. In a particularly preferred such aspect in which avidin- or streptavidin-coupled magnetic, paramagnetic or superparamagnetic beads are used as the solid support, the nucleic acid (*e.g.*, cDNA)-containing beads are segregated from the supernatants using a magnet (such as a Magna-Sep Magnetic Particle Separator; Life Technologies, Inc.) and the supernatants are withdrawn using a pipette. Prior to their release from the solid support, the immobilized nucleic acid molecules are

preferably washed one or more times, for example with one of the buffered salt solutions described above, to more fully remove unwanted materials.

Once the contaminants have been fully removed, the nucleic acid (*e.g.*, cDNA) molecules may be released from the solid support by contacting the support with an endonuclease, which may be a restriction endonuclease, that specifically recognizes the sequence engineered into the primer-adaptor molecule as described above, under conditions favoring the cleavage of the recognition sequence. In a particularly preferred aspect of the invention in which a *NotI* and/or *I-CeuI* recognition sequence is engineered into the primer-adaptor molecule (and is thus contained in the newly synthesized nucleic acid (*e.g.*, cDNA) molecules), the solid support is contacted with a solution comprising *NotI* and/or *I-CeuI*. Of course, the choice of restriction enzyme used to release the nucleic acid molecules from the solid support will depend upon the specific recognition site engineered into the primer-adaptor molecule and the possibility of that recognition site being present in the nucleic acid molecules. Preferred conditions for release of the nucleic acid molecules (*e.g.*, cDNA or cDNA libraries) from the solid support include incubation at about 20°C to about 40°C, preferably at about 25°C to about 39°C, more preferably about 30°C to about 37°C, and most preferably about 37°C, for about 30-180 minutes, preferably about 60-150 minutes, and most preferably about 120 minutes. Following their release from the solid support, the nucleic acid molecules (*e.g.*, cDNA molecules or cDNA libraries) may be processed and further purified in accordance with the invention, or by techniques that are well-known in the literature (*see, e.g.*, Gubler, U., and Hoffman, B.J., *Gene* 25:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol.* 152:316-325 (1987); Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1987)), and others that will be familiar to one of ordinary skill in the art.

Kits

The present invention also provides kits for use in production and isolation of nucleic acid molecules (*e.g.*, cDNA molecules or libraries). Kits according to this aspect of the invention comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more containers, such as vials, tubes, ampules, bottles and the like, wherein a first container contains one or more primer-adaptor nucleic acid molecules, which are preferably biotinylated primer-adaptor nucleic acid molecules. In other aspects, the kits of the invention may further comprise one or more additional containers containing a hapten-coupled solid support, which may be any of the above-described solid supports and which is most preferably avidin- or streptavidin-coupled magnetic, paramagnetic or superparamagnetic beads. In additional aspects, the kits of the invention may further comprise one or more additional containers containing, for example, one or more nucleotides (*e.g.*, dNTPs, ddNTPs or derivatives thereof) or one or more polypeptides (*e.g.*, enzymes) having reverse transcriptase activity and/or polymerase activity, preferably any of those enzymes described above. Such nucleotides or derivatives thereof may include, but are not limited to, dUTP, dATP, dTTP, dCTP, dGTP, dITP, 7-deaza-dGTP, α -thio-dATP, α -thio-dTTP, α -thio-dGTP, α -thio-dCTP, ddUTP, ddATP, ddTTP, ddCTP, ddGTP, ddITP, 7-deaza-ddGTP, α -thio-ddATP, α -thio-ddTTP, α -thio-ddGTP, α -thio-ddCTP or derivatives thereof, all of which are available commercially from sources including Life Technologies, Inc. (Rockville, Maryland), New England BioLabs (Beverly, Massachusetts) and Sigma Chemical Company (Saint Louis, Missouri). Additional kits according to the invention may comprise one or more additional containers containing one or more endonucleases or restriction enzymes used for release of the nucleic acid molecules (*e.g.*, cDNA molecules or cDNA libraries) from the solid support. The kits encompassed by this aspect of the present

invention may further comprise additional reagents (*e.g.*, suitable buffers) and compounds necessary for carrying out nucleic acid reverse transcription and/or polymerization protocols.

Uses

The present invention can be used in a variety of applications requiring rapid production and isolation of nucleic acid molecules. The invention is particularly suited for isolation of mRNA or polyA⁺ RNA molecules, for isolation of desired nucleic acid molecules from a population of nucleic acid molecules, and for production of nucleic acid molecules (particularly full-length cDNA molecules from small amounts of mRNA).

The invention is also directed to methods for the amplification of a nucleic acid molecule, and to nucleic acid molecules amplified by to these methods. According to this aspect of the invention, a nucleic acid molecule may be amplified (*i.e.*, additional copies of the nucleic acid molecule prepared) by amplifying the nucleic acid molecule (*e.g.*, a cDNA molecules) of the invention according to any amplification method that is known in the art. Particularly preferred amplification methods according to this aspect of the invention include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822). Most preferred are those methods comprising one or more PCR amplifications.

The invention is also directed to methods that may be used to prepare recombinant vectors which comprise the nucleic acid molecules or amplified nucleic acid molecules of the present invention, to host cells which comprise these recombinant vectors, to methods for the production of a recombinant

polypeptide using these vectors and host cells, and to recombinant polypeptides produced using these methods.

Recombinant vectors may be produced according to this aspect of the invention by inserting, using methods that are well-known in the art, one or more of the nucleic acid molecules or amplified nucleic acid molecules prepared according to the present methods into a vector * * * * The vector used in this aspect of the invention may be, for example, a phage or a plasmid, and is preferably a plasmid. Preferred are vectors comprising *cis*-acting control regions to the nucleic acid encoding the polypeptide of interest. Appropriate *trans*-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors are expression vectors that provide for specific expression of the cDNA molecule or nucleic acid molecule of the invention, which vectors may be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker such as a tetracycline or ampicillin resistance gene for culturing in a bacterial host cell. Prior to insertion into such an expression vector, the nucleic acid molecules (e.g., cDNA molecules) or amplified nucleic acid molecules of the invention should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters. Other suitable promoters will be known to the skilled artisan.

Among vectors preferred for use in the present invention include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from Invitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2 and pSV SPORT1, available from Life Technologies, Inc. Other suitable vectors will be readily apparent to the skilled artisan.

Representative host cells that may be used according to the invention include, but are not limited to, bacterial cells, yeast cells, plant cells and animal cells. Preferred bacterial host cells include *Escherichia* spp. cells (particularly *E. coli* cells and most particularly *E. coli* strains DH10B and Stbl2), *Bacillus* spp. cells (particularly *B. subtilis* and *B. megaterium* cells), *Streptomyces* spp. cells, *Erwinia* spp. cells, *Klebsiella* spp. cells and *Salmonella* spp. cells (particularly *S. typhimurium* cells). Preferred animal host cells include insect cells (most particularly *Spodoptera frugiperda* Sf9 and Sf21 cells and *Trichoplusa* High-Five cells) and mammalian cells (most particularly CHO, COS, VERO, BHK and human cells). These and other suitable host cells are available commercially, for example from Life Technologies, Inc., American Type Culture Collection and Invitrogen.

In addition, the invention provides methods for producing a recombinant polypeptide, and polypeptides produced by these methods. According to this aspect of the invention, a recombinant polypeptide may be produced by culturing any of the above recombinant host cells under conditions favoring production of a polypeptide therefrom, and isolation of the polypeptide. Methods for culturing recombinant host cells, and for production and isolation of polypeptides therefrom, are well-known to one of ordinary skill in the art.

In other applications, the methods of the invention may be used to generate a gene-specific cDNA library from a complex population of poly A+

RNA. The methods of the invention, in combination with polymorphism analysis methods such as AFLP, also facilitate rapid and direct identification of transcriptional differences between two different DNA populations. Additionally, the primer-adapter used in the invention can be designed to contain a regulatory sequence, such as a promoter, enhancer or other regulatory region. In one such aspect, a promoter for T7 or SP6 RNA polymerase may be engineered into the primer-adapter, thereby enabling the production of additional copies of the original mRNA for use in amplification or subtraction. Furthermore, the methods of the invention can be used to isolate poly A⁺ RNA from total RNA, such as from cells, tissues, organs or organisms, or to generate a cDNA library directly from total RNA. In the latter application, the invention is particularly useful when the mRNA of interest represents only a minute fraction of the total RNA; by the invention, this low-level mRNA may be rapidly and efficiently isolated from the background of total RNA and may then be rapidly and efficiently reverse transcribed into single-stranded or double-stranded cDNA molecules for a variety of purposes such as cloning and/or amplification.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

Example 1: *Production and Isolation of cDNA Molecules*

First and second strand cDNA synthesis reactions were conducted as described in the instruction manual for the SUPERScript Plasmid System (Life

Technologies, Inc., Rockville, Maryland), except that 50-5000 ng of mRNA was used as starting material to produce a library of $>10^6$ clones. The primer-adaptor used in cDNA synthesis contained four biotin (B) residues:

B-GACT (-B) AGT (-B)T(-B)CTAGATCGCGAGCGGCCGCCCC(T₁₅) (SEQ ID NO:1).

Briefly, 1 μ g of the biotinylated primer-adaptor was used to prime first strand synthesis for 60 minutes, in a solution containing 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 500 μ M each of dATP, dCTP, dGTP and dTTP, 50 μ M/ml Bio-p-A and 10,000 to 50,000 units/ml SuperScript II reverse transcriptase (Life Technologies, Inc.). Second strand synthesis was performed for two hours at 16°C using methods described previously (Okayama, H., and Berg, P., *Mol. Cell. Biol.* 2:161 (1982); Gubler, U., and Hoffman, B.J., *Gene* 25:263 (1983); D'Alessio, J.M., *et al.*, *FOCUS* 9:1 (1987)), in a solution containing 25 mM TRIS-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 10 mM (NH₄)₂SO₄, 0.15 mM B-NAD⁺, 250 μ M each of dATP, dCTP, dGTP and dTTP, 1.2 mM DTT, 65 units/ml DNA ligase, 250 units/ml DNA polymerase I and 13 units/ml RNase H.

During the final 30 min of the two-hour second strand cDNA synthesis reaction, streptavidin paramagnetic beads were prepared. Briefly, paramagnetic beads (Life Technologies, Inc.) were resuspended and 150 μ l of bead suspension was placed into a microcentrifuge tube for each reaction. The tubes were then placed into a Magna-Sep Magnetic particle Separator (magnet) for two minutes, and supernatant removed by aspiration. The beads were then washed by adding 100 μ l of TE buffer (10 mM TRIS-HCl (pH 7.5), 1 mM EDTA) to each tube, resuspending beads, and removing supernatant after two minutes as described above. Following washing, the beads were resuspended in 160 μ l of Binding Buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 M NaCl) and held at 25°C until use in isolating cDNA.

After incubating the second strand cDNA synthesis reaction mixtures with T4 DNA polymerase, the tubes were placed on ice and the reaction terminated by the addition to each tube of 10 μ l of 0.5 M EDTA. The biotinylated cDNA molecules were then isolated by contacting the solution with the streptavidin-coupled paramagnetic beads. Briefly, 160 μ l of the beads prepared as described above were added to the cDNA reaction mixture tubes, and the tubes gently mixed and incubated for 60 minutes at room temperature. Tubes were then inserted into the magnet for two minutes, after which supernatants were removed and discarded. The beads were then washed by gentle resuspension with 100 μ l of wash buffer (10 mM TRIS-HCl (pH 7.5), 1 mM EDTA, 500 mM NaCl), followed by re-insertion into the magnet. After two minutes, supernatants were removed and discarded and the washing step repeated. Following the second wash, beads were resuspended in 100 μ l of wash buffer, transferred into fresh tubes, and washed twice as above (with five minute exposures to the magnet).

Following the second five-minute wash, supernatant was removed and discarded and cDNA molecules were removed from the beads by incubation with *NotI*. Briefly, 50 μ l of *NotI* solution (41 μ l of autoclaved distilled water, 5 μ l of REact 3 buffer (500 mM TRIS-HCl (pH 8.0), 100 mM MgCl₂, 1 M NaCl) and 4 μ l of *NotI*) were added to each reaction tube and tubes mixed by gentle pipetting. Tubes were incubated for two hours at 37°C, then inserted into the magnet for two minutes. Supernatants containing the cDNA molecules were withdrawn into a fresh tube, and the beads gently resuspended in 20 μ l of TE buffer, re-inserted into the magnet for two minutes, and supernatants from this wash combined with those containing the cDNA molecules from above. To each tube containing pooled supernatants, 70 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the tubes vortexed thoroughly and centrifuged at room temperature for five minutes at 14,000 x g. Following centrifugation, 65 μ l of the upper, aqueous layer were removed from each tube and transferred into fresh

microcentrifuge tubes, and 32 μ l of 7.5 M ammonium acetate, 1 μ l (20 μ g) of Glycogen and 250 μ l of cold (-20°C) absolute ethanol were added to each tube. Tubes were then mixed and stored on dry ice or at -70°C for 15 minutes, then centrifuged for 30 minutes at 14,000 x g at 4°C. Supernatants were removed and discarded, 100 μ l of 70% ethanol were added to the pellets and the tubes were centrifuged for two minutes at 14,000 x g at room temperature. Supernatants were removed and discarded, and the pellets were dried in a speed-vac and then dissolved in TE buffer (10 μ l for 50-200 ng of input mRNA, or 100 μ l for 200-5000 ng of input mRNA). Final cDNA yields were determined by Cerenkov counting.

Example 2: *Vector Ligation of cDNA and Introduction into Host Cells*

From 10 to 50 ng of the cDNA was ligated into a vector (*e.g.*, pCMVSPORT) and this ligation introduced into *E. coli* by transformation as described in the SUPERScript Plasmid System manual (Life Technologies, Inc.), except the cloning vector was pre-digested with *Not*I and *Sma*I. In one such ligation, 50 ng of vector was ligated to the cDNA in a 1.5 ml microcentrifuge tube with 4 μ l of 5X T4 DNA ligase buffer (250 mM TRIS-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG-8000) and 1 μ l of T4 ligase (1 unit) at 4°C for 16 hours.

Example 3: *cDNA Yield Comparisons*

To examine the efficiency and yield of cDNA synthesis by the methods of the invention, cDNA was produced as described above and the amounts produced were compared to those obtained using an alternative commercially available system (SUPERScript Plasmid System; Life Technologies, Inc., Rockville, Maryland). Briefly, after introducing the pCMV SPORT-cDNA ligations into

MAX EFFICIENCY DH5 α TM and ELECTROMAX® DH10B cells, the cells were plated onto ampicillin-containing plates to determine transformation efficiencies. The cDNA inserts were sized by using the SP6 and T7 promoter primers and 40 cycles of PCR on 48 randomly chosen colonies for each experiment.

Table 1 shows a comparison of the cDNA yields obtained by the methods of the present invention to those obtained using the SuperScript Plasmid System.

Table 1. Comparison of the Invention to the SUPERScript Plasmid System.

System Tested	Input mRNA per reaction (ng)	Yield of cDNA (ng)	Transformants per ligation (MAX EFFICIENCY DH5 α TM)	Avg. Insert Size, basepairs (Range)
Present Invention	1000	117	1.6×10^4	1210 (580-2040)
	5000	619	2.5×10^4	1030 (220-1810)
SUPER-SCRIPT Plasmid System	1000	27	1.8×10^4	840 (450-1400)
	5000	231	2.0×10^4	1280 (240-2080)

These results demonstrate that the present invention produces about three- to four-fold greater yields of cDNA than the SUPERScript Plasmid System. Furthermore, the present invention demonstrates approximately equivalent transformation efficiencies and average insert sizes to those obtained with the SUPERScript Plasmid System. Thus, the present invention provides methods for the rapid and efficient production of full-length cDNA molecules without the use of time-consuming and yield-reducing cDNA size fractionation steps.

Example 4: *Production and Isolation of cDNA Using Varying Amounts of Input mRNA*

Having demonstrated that the methods of the invention produce cDNA rapidly and efficiently, the efficacy of the invention in producing cDNA from varying amounts of input mRNA was examined. In these studies, the amount of

input mRNA was varied from 5 ng to 1 µg and the cDNA yield, transformation efficiency and average insert size determined as above. Results are shown in Table 2.

Table 2. Yield of cDNA Using Different Amounts of Input mRNA.

Input mRNA per reaction (ng)	Yield of cDNA (ng)	Transformants per ligation (ELECTROMAX® DH10B)	Avg. Insert Size, basepairs (Range)
5	2	2.7×10^5	600 (200-2000)
50	11	5.1×10^6	650 (280-1600)
200	55	8.0×10^6	930 (340-2200)
1000	389	7.5×10^6	1300 (150-2900)

These results demonstrate that the present invention is capable of producing large cDNA libraries (*i.e.*, $>10^5$ clones) from as little as 5 ng of input mRNA. Previously, PCR (a process that biases the cDNA library) was the only method that would have enabled the production of cDNA libraries from this small amount of RNA. Together with those above, these results indicate that the invention is capable of rapidly and efficiently producing high-quality, full-length cDNA molecules from varying quantities of input mRNA, including those that show a low level of expression and thus represent only a small fraction of the polyA⁺ or total RNA pools.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such

modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Gruber, Christian E.
Jessee, Joel A.
- (ii) TITLE OF INVENTION: Methods for Production and Purification
of Nucleic Acid Molecules
- (iii) NUMBER OF SEQUENCES: 1
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
 - (B) STREET: 1100 New York Avenue, N.W., Suite 600
 - (C) CITY: Washington
 - (D) STATE: DC
 - (E) COUNTRY: USA
 - (F) ZIP: 20005
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: (To be assigned)
 - (B) FILING DATE: (Herewith)
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/046,219
 - (B) FILING DATE: 12-MAY-1997
 - (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Esmond, Robert W.
- (B) REGISTRATION NUMBER: 32,893
- (C) REFERENCE/DOCKET NUMBER: 0942.4350000

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 202-371-2600
- (B) TELEFAX: 202-371-2540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: modified_base
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /mod_base= OTHER
- /note= "Guanidine at position 1 is biotinylated"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
 - (B) LOCATION: 4
 - (D) OTHER INFORMATION: /mod_base= OTHER
- /note= "Thymidine at position 4 is biotinylated"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
 - (B) LOCATION: 7
 - (D) OTHER INFORMATION: /mod_base= OTHER
- /note= "Thymidine at position 7 is biotinylated"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
 - (B) LOCATION: 8
 - (D) OTHER INFORMATION: /mod_base= OTHER
- /note= "Thymidine at position 8 is biotinylated"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GACTAGTTCT AGATCGCGAG CGGCCGCCCT TTTTTTTTTT TTTT

In pertinent part, the disclosure of United States provisional application serial number 60/122,395, filed March 2, 1999, reads as follows:

The present invention alleviates, prevents, reduces or substantially reduces internal priming thereby providing improvements in cDNA and cDNA library construction. Accordingly, the present invention greatly facilitates gene discovery by providing cDNA libraries containing a greater percentage of full-length genes.

The present invention therefore relates to synthesizing a cDNA molecule or molecules from an mRNA template or population of mRNA templates under conditions sufficient to increase the total amount of cDNA produced, increase the length of the cDNA molecules produced, and/or increase the amount or percentage of full-length cDNA molecules produced. In accordance with the invention, any conditions which inhibit, prevent, reduce or substantially reduce internal priming may be used. Such conditions preferably include but are not limited to optimizing primer concentrations, optimizing reaction temperatures and/or optimizing primer length or specificity. Such result may also be accomplished in accordance with the invention by optimizing the reverse transcription reaction, preferably by inhibiting or preventing reverse transcription until optimum or desired reaction conditions are achieved.

Conventional methods for constructing cDNA libraries use a molar ratio of oligo(dT) primer/mRNA template of 15:1 for first strand cDNA synthesis. The use of such excess amounts of oligo(dT) primer allows internal priming of one or more primers to one or more of the mRNA templates in the reaction. According to a preferred aspect of the present invention, the amount of oligo(dT) primer is reduced for synthesis of first strand cDNA to inhibit, prevent, reduce or substantially reduce internal priming. Preferred molar ratios of primer to template range from about 12:1; 10:1; 9:1; 8:1; 7:1; 6:1; 5:1; 4:1; 3:1; 2:1; 1:1; 1:2; 1:3; 1:4; 1:5; 1:6; 1:7; 1:8; 1:9; 1:10 and 1:12. Preferably, molar ratios of primer (e.g., oligo(dT)) to template (e.g., mRNA) range from about 5:1 to about 1:20, although lower molar ratios of primer to template may be used in accordance with the invention. Specifically, molar ratios of primer

to template may be below about 1:10; 1:15; 1:20; 1:25; 1:50; 1:75; and 1:100. Preferably, ranges of molar ratios are below about 5:1; 4:1; 3:1; 2:1; 1:1; 1:2; 1:3; 1:4; and 1:5. Most preferably, ratios of primer to template range from about 10:1 to 1:10; 5:1 to 1:10; 4:1 to 1:10; 3:1 to 1:10; 2.5:1 to 1:10; 2:1 to 1:10; 1.5:1 to 1:10; and 1:1 to 1:10. The optimum ratios of primer to template may vary depending on the primer, mRNA, reverse transcription enzyme and reaction conditions (annealing temperature, buffering salts, etc.). The desired primer to template ratios can be readily determined by one skilled in the art.

In conventional methods of cDNA library construction, annealing or hybridizing primer to template is not carried out at a temperature which prevents, inhibits, reduces or substantially reduces internal priming. Typically, the mixture (e.g., mRNA and oligo(dT) primer) is chilled on ice after denaturation or heating. This process typically causes annealing or hybridization of the primer to internal sites. According to a preferred aspect of the present invention, the temperature during the annealing or hybridization between the primer and the template is maintained so that internal priming is inhibited, prevented, reduced or substantially reduced. In accordance with the invention, such a result is accomplished by carrying out primer annealing or hybridization at higher temperatures. Such conditions may also reduce the formation of mRNA secondary structures during cDNA synthesis. Preferably, temperatures for annealing or hybridizing primers to the templates range from about 10°C to about 90°C; more preferably about 10°C to about 80°C; still more preferably about 20°C to about 75°C; more preferably about 25°C to about 75°C; still more preferably about 30°C to about 65°C; still more preferably about 37°C to about 60°C; still more preferably about 40°C to about 60°C; still more preferably about 45°C to about 60°C; still more preferably about 45°C to about 55°C; and most preferably about 45°C to about 65°C. The temperature used may vary depending on the type and amount of primer and template and depending on the temperature optimum of the reverse transcription enzyme. The optimum temperature or temperature ranges can be readily determined by one skilled in the art.

Conventional methods for cDNA synthesis typically requires the use of oligo(dT) primers of a particular length (12-18 bases or mer). Such primer length, however, lowers specificity of the primer thereby allowing internal priming. Thus, the invention also relates to increasing specificity of the primers to prevent, inhibit, reduce or substantially

reduce internal priming. In a preferred aspect, primer specificity is increased by increasing the length of the primer. Thus, for cDNA synthesis, longer oligo(dT) primers may be used in accordance with the invention. Preferably, primer length ranges from about 20 to about 100 bases, about 20 to about 75 bases, about 20 to about 60 bases, and about 20 to about 50 bases; more preferably about 20 to about 45 bases; more preferably about 20 to about 40 bases; and most preferably about 25 to about 35 bases. In a preferred aspect, the length of the primers are greater than 19 bases; more preferably greater than about 20 bases; more preferably greater than about 25 bases; and still more preferably greater than about 30 bases. Such primer lengths refer to the length of the primers which anneal or hybridize to the template. Optimum length and content (nucleotide sequence) of the primers may vary depending on the type of template, the desired reaction conditions, and the reverse transcription enzyme. In accordance with the invention, additional sequences and/or modified nucleotides may be included in the primers of the invention. For example, additional sequences (which do not necessarily anneal or hybridize to the template) may be included in the primers of the invention to assist in cDNA synthesis including sequences comprising one or more restriction endonuclease sites, one or more derivative nucleotides (e.g., hapten containing nucleotides such as biotinylated nucleotides), and the like. The type and length of the primers used in accordance with the invention can be readily determined by one or more skilled in the art.

Conventional cDNA synthesis methods do not control or vary activity of the reverse transcription enzyme to optimize the reverse transcription reaction. In accordance with the invention, the activity of the reverse transcriptase is preferably controlled to start synthesis at a desired time in the reaction. In a preferred aspect, reverse transcriptase activity is inhibited or prevented until optimum or desired reaction conditions are achieved. Such a result is accomplished in accordance with the invention by the use of inhibitors (such as antibodies or antibody fragments) which inhibit reverse transcriptase activity. Such reverse transcriptase inhibitors prevent or inhibit reverse transcriptase activity at low temperatures such that internal priming is prevented, inhibited, reduced or substantially reduced. In accordance with the invention, such

inhibitors preferably prevent reverse transcriptase activity below 35°C, below 40°C, below 45°C, below 50°C, below 55°C, below 60°C, below 65°C, below 70°C, below 75°C, below 80°C, below 85°C and below 90°C. Depending on the thermostability of the enzyme having reverse transcriptase activity, the inhibitor may be designed to inhibit activity of the enzyme at a point at or near the temperature optimum for the enzyme of interest. Preferably, the inhibitor is inactivated at a temperature below or near the temperature optimum of the enzyme used, thereby allowing reverse transcription to take place. Thus, the invention generally relates to the use of reverse transcriptase inhibitors in cDNA synthesis. The type and amount of inhibitor may vary depending on the type and amount of reverse transcription enzyme and depending on the reaction conditions to be used. The type of inhibitor and conditions used with such inhibitor can be readily determined by one of ordinary skill in the art.

In accordance with the invention, any one or a combination of the above improvements to cDNA synthesis may be used. Using any one or a combination of these improvements provides for improved first strand cDNA synthesis (e.g., more total cDNA, larger cDNA and/or more full-length cDNA). In accordance with the invention, the first strand cDNA molecules may be used as templates to make one or more double stranded nucleic acid molecules (e.g., double strand cDNA molecules) by incubating one or more of the first strand cDNA molecules produced by the methods of the invention under conditions sufficient to make one or more nucleic acid molecules complementary to all or a portion of the first strand cDNA molecules. Conditions for making double stranded nucleic acid molecules preferably include incubation with one or more components consisting of one or more DNA polymerases, one or more nucleotides, one or more buffering salts, and one or more primers. In another aspect of the invention, such conditions are modified to provide an increase in the total amount of double stranded cDNA produced, an increase in the length or size of the double stranded cDNA molecule produced, and/or an increase in percentage full-length double stranded cDNA molecule produced. Preferably, such conditions relate to optimization of ribonuclease (RNase) digestion after first strand cDNA synthesis. During first strand cDNA synthesis, if a full-length cDNA molecule complementary to the mRNA template is not made, a

single stranded mRNA containing the cap structure will be present at the 5' end of the mRNA of the mRNA/cDNA hybrid. If a full-length cDNA is produced, a double stranded mRNA/cDNA hybrid is produced with no single stranded mRNA present. Preferably, such digestion conditions are optimized so that the single stranded mRNA of the mRNA/cDNA double stranded molecules formed during first strand cDNA synthesis is subject to RNase digestion. In this manner, cap structure from mRNA/cDNA hybrids which are not full-length are removed while full-length mRNA/cDNA hybrids will retain the cap structure. Thus, cap capture can be used to select for full-length molecules and select against molecules which are not fulllength. In a preferred aspect, the conditions are such that the single stranded mRNA of the mRNA/cDNA hybrid is digested or degraded while the mRNA of the double stranded mRNA/cDNA hybrid is not degraded or not substantially degraded. Thus, such RNase digestion is conducted under conditions such that second strand synthesis is not substantially adversely affected. That is, second strand synthesis in accordance with the invention produces larger double stranded cDNA molecules compared to conventional techniques. Conventional RNase I conditions typically range from 25 u/μg to 40 u/μg mRNA at 37°C and RNase A conditions typically are 1000 ng/μg mRNA at 37°C. Using conventional RNase digestion, the average size of double stranded cDNA molecules produced is about 200 bases. According to the present invention the average size of double stranded cDNA molecules produced is preferably greater than about 300 bases, greater than about 400 bases, greater than about 500 bases, greater than about 600 bases, greater than about 700 bases, greater than about 800 bases, greater than about 900 bases, greater than about 1 kilobase, greater than about 1.5 kilobases, and greater than about 2 kilobases. In one embodiment of the invention, the concentration of the ribonuclease, the type of ribonuclease and reaction conditions are optimized to improve double stranded cDNA synthesis in accordance with the invention. Preferred ribonucleases for use in ribonuclease digestions include ribonuclease A (RNase A) and/or ribonuclease I (RNase I). Generally, lower temperatures (about 4°C to about 50°C) and higher salt concentrations (about 5 mM to about 5 M) will assist in inhibiting or controlling RNase digestion in accordance with the invention. Salts used may include sodium chloride, potassium, chloride, magnesium chloride, sodium acetate etc. Additionally, lowering RNase

amounts or concentrations may be used to accomplish the desired result. Such concentrations for RNase A may range from about 0.001 ng/ μ g mRNA to about 500 ng/ μ g of mRNA and for RNase I may range from about 0.001 u/ μ g mRNA to about 500 u/ μ g mRNA. The incubation temperature, RNase concentration and salt concentration may be readily determined by one skilled in the art. In a preferred aspect, concentration of the RNase A include ranges from 0.1 ng/ μ g mRNA to 10 ng/ μ g mRNA in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at 37°C. Alternatively, the concentration of the RNase A can include ranges from 0.1 ng/ μ g mRNA to 500 ng/ μ g mRNA in 10 mM Tris, pH 7.5 buffer containing 250 mM NaCl at 25°C for 30 minutes. Preferably, concentration of the RNase I used ranges from 0.1 unit/ \sim tg mRNA to 1.0 unit/Rg mRNA in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 200 mM sodium acetate at 37°C. Alternatively, the concentration of the RNase I can be used at ranges from 1.0 unit/ μ g mRNA to 100 units/ μ g mRNA in the same buffer at 25°C for 30 minutes.

In another aspect, the invention relates to capture or binding of the cap structure (e.g., m⁷GpppN) of the mRNA before, during or after first strand cDNA synthesis. Thus, the invention relates to selection of mRNA (before first strand synthesis) or mRNA/cDNA hybrids (after or during first strand synthesis) which have the cap structure in carrying out the methods of the invention. Such selection or capture may be accomplished with any cap binding molecule such as eIF4E, eIF4E peptides, eIF4E peptide fragments (see WO 98/08865) and antibodies or antibody fragments specific for cap structure. In a preferred aspect, selection of the cap structure is accomplished after first strand synthesis. More preferably, such cap capture occurs after ribonuclease digestion in accordance with the methods of the invention. For example, mRNA/cDNA hybrids subjected to ribonuclease digestion are captured and then used for second strand cDNA synthesis according to the invention.

Thus, the present invention is generally directed to methods of synthesizing nucleic acid molecules. The present invention is more specifically directed to methods of making one or more nucleic acid molecules, especially cDNA molecules or cDNA libraries, comprising mixing one or more nucleic acid templates (preferably mRNA, poly A RNA or a population of mRNA molecules) with at least one polypeptide having

reverse transcriptase activity, and incubating the mixture under conditions sufficient to make one or more first nucleic acid molecules (e.g., first strand cDNA) complementary to all or a portion of the one or more nucleic acid templates. In accordance with the invention, such conditions provide for an increased total amount of nucleic acid molecule (cDNA) produced, compared to conventional procedures which do not employ the improved modifications or conditions of the invention. The invention also provides for an increase of length or average size of the nucleic acid molecules (cDNA) produced and/or an increase in the percentage or amount of full-length nucleic acid molecules (cDNA) produced, compared to conventional procedures which do not employ the improved :modifications or conditions of the invention. Determining the amount, length and full-length content of the cDNA produced can be determined by conventional techniques well known in the art and as described herein. The percentage or average percentages of full-length cDNA in cDNA libraries produced in accordance with the invention are preferably above about 15%, more preferably above about 20%, more preferably above about 25%, more preferably above about 30%, more preferably above about 40%, more preferably above about 50%, more preferably above about 60%, more preferably above about 70%, more preferably above about 80% and most preferably above about 90%. Such full-length percentages are preferably determined by random selection of a portion of the clones of the cDNA library of interest (e.g., 100 to 1000 clones), sequencing the clones and comparing the sequences to known sequence data bases.

In preferred aspects of the invention, the improved results of the invention are preferably accomplished by one or a combination of modifications to the conditions for nucleic acid or cDNA synthesis. Such conditions preferably include modifications for improving first strand cDNA synthesis and/or improving second strand cDNA synthesis.

In a preferred aspect, the invention specifically relates to methods of making one or more double stranded cDNA molecules comprising incubating one or more mRNA molecules (preferably a population of mRNA molecules) with one or more primers of the invention at temperatures and primer concentrations to prevent, inhibit, reduce or substantially reduce internal priming prior to or during first strand cDNA synthesis.

Such reaction is preferably conducted in the presence of one or more inhibitors of reverse transcriptase activity in accordance with the invention. Ribonuclease digestion is preferably conducted before second strand cDNA synthesis and at ribonuclease concentrations sufficient to increase the length, amount and/or size of double stranded cDNA molecules produced during second strand synthesis. In accordance with the invention, cap capture is preferably accomplished during or after the ribonuclease digestion.

The invention is also directed to nucleic acid molecules and cDNA molecules or populations of cDNA molecules (single or double-stranded) produced according to the above-described methods and to vectors (particularly expression vectors) comprising these nucleic acid molecules and cDNA molecules. The invention also relates to host cells containing such cDNA molecules and/or vectors.

The invention is also directed to kits for use in the methods of the invention. Such kits can be used for making single or double-stranded nucleic acid molecules. The kits of the invention comprise a carrier, such as a box or carton, having therein one or more containers, such as vials, tubes, bottles and the like. Such kits may comprise at least one component selected from the group consisting of primers (preferably primers having higher specificity and most preferably oligo(dT) primers having a length equal to or greater than 20 bases), one or more polypeptides having reverse transcriptase activity (reverse transcriptases and DNA polymerases), one or more inhibitors of reverse transcription (e.g., antibodies and antibody fragments directed against polypeptides having RT activity), one or more cap binding molecules (e.g., antibodies or antibody fragments directed against cap structure), nucleic acid synthesis reaction buffers, one or more nucleotides, one or more vectors, and instructions for carrying out the methods of the invention.

The invention also relates to compositions for use in the invention or made while carrying out the methods of the invention. Such compositions may comprise at least one primer (e.g., oligo(dT) or derivatives thereof) and at least one template in a sample or reaction mixture in amounts or ratios in accordance with the invention. Such composition may further comprise one or more polypeptides having reverse transcriptase

activity, one or more reverse transcription inhibitors (e.g., anti-RT antibodies or fragments thereof), one or more nucleotides, one or more cap binding molecules (e.g., anti-cap antibodies for fragments thereof), one or more buffering salts and the like. Such compositions may also be maintained at a temperature to avoid internal priming in accordance with the invention.

The compositions of the invention may also comprise amounts of ribonuclease in accordance with the invention. Such compositions may further comprise at least one component selected from one or more mRNA/cDNA hybrids, one or more nucleotides, one or more polypeptides having reverse transcriptase activity, one or more buffering salts, one or more cap binding molecules (e.g., anti-cap antibodies or fragments thereof) and the like.

The invention also relates to one or more antibodies (monoclonal and polyclonal) and fragments thereof for use in the methods, compositions and kits of the invention. Such antibodies, include anti-cap and/or anti-RT antibodies and antibody fragments.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in the art in view of the following drawings and description of the invention.

* * *

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions:

In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Internal priming as used herein refers to hybridization or annealing of one or more primers at one or more sites within one or more mRNA molecules other than at the poly A tail located at the 3' termini of the mRNA molecule.

Library as used herein refers to a set of nucleic acid molecules (circular or linear) which is representative of all or a portion or significant portion of the DNA content of an organism (a "genomic library"), or a set of nucleic acid molecules representative of all or a portion or significant portion of the expressed genes (a "cDNA library") in a cell, tissue, organ or organism. Such libraries may or may not be contained in one or more vectors.

Vector as used herein refers to a plasmid, cosmid, phagemid or phage DNA or other DNA molecule which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which DNA may be inserted in order to bring about its replication and cloning. The vector may further contain one or more markers suitable for use in the identification of cells transformed with the vector. Markers, for example, include but are not limited to tetracycline resistance or ampicillin resistance. Such vectors may also contain one or more recombination sites, one or more termination sites, one or more origins of replication, and the like.

Primer as used herein refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a DNA molecule. Preferred primers for use in the invention include oligo(dT) primers or derivatives or variants thereof.

Oligonucleotide as used herein refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are joined by a phosphodiester bond between the 3' position of the deoxyribose or ribose of one nucleotide and the 5' position of the deoxyribose or ribose of the adjacent nucleotide.

Template as used herein refers to double-stranded or single-stranded nucleic acid molecules which are to be amplified, synthesized or sequenced. In the case of a double-stranded molecules, denaturation of its strands to form a first and a second strand is preferably performed before these molecules may be amplified, synthesized or sequenced, or the double stranded molecule may be used directly as a template. For

single stranded templates, a primer, complementary to a portion of the template is hybridized or annealed under appropriate conditions and one or more polymerases or reverse transcriptases may then synthesize a nucleic acid molecule complementary to all or a portion of said template. The newly synthesized molecules, according to the invention, may be equal or shorter in length than the original template.

Incorporating as used herein means becoming a part of a DNA and/or RNA molecule or primer.

Amplification as used herein refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA and/or RNA molecule or primer thereby forming a new molecule complementary to a template. The formed nucleic acid molecule and its template can be used as templates to synthesize additional nucleic acid molecules. As used herein, one amplification reaction may consist of many rounds of replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a DNA molecule.

Nucleotide as used herein refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes ribonucleoside triphosphate ATP, UTP, CTG, GTP and deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, [α S]dATP, 7-deaza-dGTP, 7-deaza-dATP, and biotinylated or haptenylated nucleotides. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by well known techniques. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

Hybridization or **annealing** as used herein refers to base pairing of two complementary single-stranded nucleic acid molecules (RNA and/or DNA) to give a double-

stranded molecule. As used herein, two nucleic acid molecules may be hybridized or annealed, although the base pairing is not completely complementary. Accordingly, mismatched bases do not prevent hybridization or annealing of two nucleic acid molecules provided that appropriate conditions, well known in the art, are used. In the present invention, the term hybridization or annealing preferably refers to hybridization of one or more primers (e.g., oligo(dT) or derivatives thereof) to one or more templates (e.g., mRNA).

Host cell as used herein refers to any prokaryotic or eukaryotic cell that is the recipient of a replicable expression vector or cloning vector. The terms "host" or "host cell" may be used interchangeably herein. For examples of such hosts, see Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Preferred prokaryotic hosts include, but are not limited to, bacteria of the genus *Escherichia* (e.g., *E. coli*), *Bacillus*, *Staphylococcus*, *Agrobacter* (e.g., *A. tumefaciens*), *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, *Caryophanon*, etc. The most preferred prokaryotic host is *E. coli*. Bacterial hosts of particular interest in the present invention include *E. coli* strains K12, DH10B, DH5 α , Stb12 and HB101, and others available from Life Technologies, Inc. Preferred eukaryotic hosts include, but are not limited to, fungi, fish cells, yeast cells, plant cells and animal cells. Particularly preferred animal cells are insect cells such as *Drosophila* cells, *Spodoptera* Sf9, Sf21 cells and *Trichoplusia* High-Five cells; nematode cells such as *C. elegans* cells; and mammalian cells such as COS cells, CHO cells, VERO cells, 293 cells, PERC6 cells, BHK cells and human cells.

Expression vector as used herein refers to a vector which is capable of enhancing the expression of a gene or portion of a gene which has been cloned into it, after transformation or transfection into a host cell. The cloned gene is usually placed under the control (i.e., operably linked to) certain control sequences such as promoter sequences. Such promoters include but are not limited to phage lambda P_L promoter, and the *E. coli lac*, *trp* and *tac* promoters. Other suitable promoters will be known to the skilled artisan.

The nucleic acid templates suitable for reverse transcription according to the invention include any nucleic acid molecule or populations of nucleic acid molecules

(preferably one or more RNA molecules (e.g., one or more mRNA molecules or poly A⁺ RNA molecules, and more preferably a population of mRNA molecules) or one or more DNA molecules), particularly those derived from a cell or tissue. In a preferred aspect, a population of mRNA molecules (a number of different mRNA molecules) are used to make a cDNA library according to the present invention.

To make the nucleic acid molecule or molecules complementary to the one or more templates, a primer (e.g., an oligo(dT) primer) and one or more nucleotides are used for nucleic acid synthesis typically in the 3' to 5' direction. Nucleic acid molecules suitable for reverse transcription according to this aspect of the invention include any nucleic acid molecule, particularly those derived from a prokaryotic or eukaryotic cell. Such cells may include normal cells, diseased cells, transformed cells, established cells, progenitor cells, precursor cells, fetal cells, embryonic cells, bacterial cells, yeast cells, animal cells (including human cells), avian cells, plant cells and the like, or tissue isolated from a plant (e.g., corn, tomato, tobacco, potato, soy bean, etc.) or an animal (e.g., human, cow, pig, mouse, sheep, horse, monkey, canine, feline, rat, rabbit, bird, fish, insect, etc.). Such nucleic acid molecules may also be isolated from viruses.

The nucleic acid molecules that are used as templates to prepare cDNA molecules according to the methods of the present invention are preferably obtained from natural sources, such as a variety of cells, tissues, organs or organisms. Cells that may be used as sources of nucleic acid molecules may be prokaryotic (bacterial cells, including but not limited to those of species of the genera *Escherichia*, *Bacillus*, *Serratia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Chlamydia*, *Neisseria*, *Treponema*, *Mycoplasma*, *Borrelia*, *Legionella*, *Pseudomonas*, *Mycobacterium*, *Helicobacter*, *Erwinia*, *Agrobacterium*, *Rhizobium*, *Xanthomonas* and *Streptomyces*) or eukaryotic (including fungi (especially yeasts), plants, protozoans and other parasites, and animals including insects (particularly *Drosophila* spp. cells), nematodes (particularly *Caenorhabditis elegans* cells), and mammals (particularly human cells)).

Mammalian somatic cells that may be used as sources of nucleic acids include blood cells (reticulocytes and leukocytes), endothelial cells, epithelial cells, neuronal

cells (from the central or peripheral nervous systems), muscle cells (including myocytes and myoblasts from skeletal, smooth or cardiac muscle), connective tissue cells (including fibroblasts, adipocytes, chondrocytes, chondroblasts, osteocytes and osteoblasts) and other stromal cells (e.g., macrophages, dendritic cells, Schwann cells). Mammalian germ cells (spermatocytes and oocytes) may also be used as sources of nucleic acids for use in the invention, as may the progenitors, precursors and stem cells that give rise to the above somatic and germ cells. Also suitable for use as nucleic acid sources are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus.

Any of the above cells, tissues and organs may be normal, diseased, transformed, established, progenitors, precursors, fetal or embryonic. Diseased cells may, for example, include those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS, HIV, HTLV, herpes, hepatitis and the like) or parasites), in genetic or biochemical pathologies (e.g., cystic fibrosis, hemophilia, Alzheimer's disease, muscular dystrophy or multiple sclerosis) or in cancerous processes. Transformed or established animal cell lines may include, for example, COS cells, CHO cells, VERO cells, BHK cells, HeLa cells, HepG2 cells, K562 cells, 293 cells, L929 cells, F9 cells, and the like. Other cells, cell lines, tissues, organs and organisms suitable as sources of nucleic acids for use in the present invention will be apparent to one of ordinary skill in the art.

Once the starting cells, tissues, organs or other samples are obtained, nucleic acid molecules (such as mRNA) may be isolated therefrom by methods that are well-known in the art (See, e.g., Maniatis, T., *et al.*, *Cell* 15:687-701 (1978); Okayama, H., and Berg, P., *Mol. Cell. Biol.* 2:161-170 (1982); Gubler, U., and Hoffinan, B.J., *Gene* 25:263-269 (1983); and Message Maker TM mRNA Isolation System available from Life Technologies, Inc.). The nucleic acid molecules thus isolated may then be used to prepare cDNA molecules and cDNA libraries in accordance with the present invention. The cDNA molecules and/or cDNA libraries produced in accordance with the invention

are preferably contained in one or more vectors. Such vectors may be introduced into one or more host cells by standard transformation or transfection techniques well known in the art. Preferred host cells include prokaryotic host cells such as cells of the genus *Escherichia*, particularly *E. coli*.

Enzymes for use in the compositions, methods and kits of the invention include any enzyme having reverse transcriptase activity. Such enzymes include, but are not limited to, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, *Tth* DNA polymerase, *Taq* DNA polymerase (Saiki, R.K., *et al.*, *Science* 239:487-491 (1988); U.S. Patent Nos. 4,889,818 and 4,965,188), *The* DNA polymerase (WO 96/10640), Tma DNA polymerase (U.S. Patent No. 5,374,553) and mutants, fragments, variants or derivatives thereof (see, e.g., commonly owned, co-pending U.S. Patent Application Nos. 08/706,702 and 08/706,706, both filed September 9, 1996, which are incorporated by reference herein in their entireties). As will be understood by one of ordinary skill in the art, modified reverse transcriptases and DNA polymerase having RT activity may be obtained by recombinant or genetic engineering techniques that are well-known in the art. Mutant reverse transcriptases or polymerases can, for example, be obtained by mutating the gene or genes encoding the reverse transcriptase or polymerase of interest by site-directed or random mutagenesis. Such mutations may include point mutations, deletion mutations and insertional mutations. Preferably, one or more point mutations (e.g., substitution of one or more amino acids with one or more different amino acids) are used to construct mutant reverse transcriptases or polymerases for use in the invention. Fragments of reverse transcriptases or polymerases may also be obtained by deletion mutation by recombinant techniques that are well-known in the art, or by enzymatic digestion of the reverse transcriptase(s) or polymerase(s) of interest using any of a number of well-known proteolytic enzymes.

Preferred enzymes for use in the invention include those that are reduced or substantially reduced in RNase H activity. Such enzymes that are reduced or

substantially reduced in RNase H activity may be obtained by mutating the RNase H domain within the reverse transcriptase of interest, preferably by one or more point mutations, one or more deletion mutations, and/or one or more insertion mutations as described above. By an enzyme "substantially reduced in RNase H activity" is meant that the enzyme has less than about 30%, less than about 25%, less than about 20%, more preferably less than about 15%, less than about 10%, less than about 7.5%, or less than about 5%, and most preferably less than about 5% or less than about 2%, of the RNase H activity of the corresponding wildtype or RNase H⁺ enzyme such as wildtype Moloney Murine Leukemia Virus (M-MLV), Avian Myeloblastosis Virus (AMV) or Rous Sarcoma Virus (RSV) reverse transcriptases. The RNase H activity of any enzyme may be determined by a variety of assays, such as those described, for example, in U.S. Patent No. 5,244,797, in Kotewicz, M.L., *et al.*, *Nucl. Acids Res.* 16:265 (1988), in Gerard, G.F., *et al.*, *FOCUS* 14(5):91 (1992), and in U.S. Patent No. 5,668,005, the disclosures of all of which are fully incorporated herein by reference.

Polypeptides having reverse transcriptase activity for use in the invention may be obtained commercially, for example from Life Technologies, Inc. (Rockville, Maryland), Pharmacia (Piscataway, New Jersey), Sigma (Saint Louis, Missouri) or Boehringer Mannheim Biochemicals (Indianapolis, Indiana). Alternatively, polypeptides having reverse transcriptase activity may be isolated from their natural viral or bacterial sources according to standard procedures for isolating and purifying natural proteins that are well-known to one of ordinary skill in the art (see, e.g., Houts, G.E., *et al.*, *J. Virol.* 29:517 (1979)). In addition, the polypeptides having reverse transcriptase activity may be prepared by recombinant DNA techniques that are familiar to one of ordinary skill in the art (see, e.g., Kotewicz, M.L., *et al.*, *Nucl. Acids Res.* 16:265 (1988); Soltis, D.A., and Skalka, A.M., *Proc. Natl. Acad. Sci. USA* 85:3372-3376 (1988)).

Preferred polypeptides having reverse transcriptase activity for use in the invention include M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase and Human Immunodeficiency Virus (HIV) reverse transcriptase, and others described in WO 98/47921 and derivatives, variants,

fragments or mutants thereof, and combinations thereof. In a further preferred embodiment, the reverse transcriptases are reduced or substantially reduced in RNase H activity, and are most preferably selected from the group consisting of M-MLV H⁻ reverse transcriptase, RSV H⁻ reverse transcriptase, AMV H⁻ reverse transcriptase, RAV H⁻ reverse transcriptase, MAV H⁻ reverse transcriptase and HIV H⁻ reverse transcriptase, and derivatives, variants, fragments or mutants thereof, and combinations thereof. Reverse transcriptases of particular interest include AMV RT and M-MLV RT, and more preferably AMV RT and M-MLV RT having reduced or substantially reduced RNase H activity (preferably AMV RT α H-/BH⁺ and M-MLV RT H⁻). The most preferred reverse transcriptases for use in the invention include SuperScript[™], SuperScript[™] II, ThermoScript[™] and ThermoScript[™] II available from Life Technologies, Inc. See generally, WO 98/47921, U.S. Patents 5,244,797 and 5,668,005, the entire contents of each of which are herein incorporated by reference.

A variety of DNA polymerases are useful in accordance with the present invention. Such polymerases include, but are not limited to, *Thermus thermophilus* (*Tth*) DNA polymerase, *Thermus aquaticus* (*Taq*) DNA polymerase, *Thermotoga neapolitana* (*Tne*) DNA polymerase, *Thermotoga maritima* (*Tma*) DNA polymerase, *Thermococcus litoralis* (*Tli* or VENT[™]) DNA polymerase, *Pyrococcus furiosus* (*Pfu*) DNA polymerase, DEEPVENT[™] DNA polymerase, *Pyrococcus woosii* (*Pwo*) DNA polymerase, *Bacillus sterothermophilus* (*Bst*) DNA polymerase, *Bacillus caldophilus* (*Bca*) DNA polymerase, *Sulfolobus acidocaldarius* (*Sac*) DNA polymerase, *Thermoplasma acidophilum* (*Tac*) DNA polymerase, *Thermus flavus* (*Tfl/Tub*) DNA polymerase, *Thermus ruber* (*Tru*) DNA polymerase, *Thermus brockianus* (DYNAZYME[™]) DNA polymerase, *Methanobacterium thermoautotrophicum* (*Mth*) DNA polymerase, *Mycobacterium* spp. DNA polymerase (*Mtb*, *Mlep*), and mutants, variants and derivatives thereof.

DNA polymerases used in accordance with the invention may be any enzyme that can synthesize a DNA molecule from a nucleic acid template, typically in the 5' to 3' direction. Such polymerases may be mesophilic or thermophilic. Mesophilic polymerases include T4 DNA polymerase, T5 DNA polymerase, T7 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III, DNA polymerase I and the like. Thermostable DNA polymerases include

Taq, *Tne*, *Tma*, *Pfu*, VENT[™], DEEPVENT[™], *Tth* and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; Barnes, W.M., *Gene* 112:29-35 (1992); Lawyer, F.C., *et al.*, *PCR Meth. Appl.* 2:275-287 (1993); Flaman, J.-M., *et al.*, *Nucl. Acids Res.* 22(15):3259-3260 (1994)).

DNA polymerases for use in the invention may be obtained commercially, for example from Life Technologies, Inc. (Rockville, Maryland), Perkin-Elmer (Branchburg, New Jersey), New England BioLabs (Beverly, Massachusetts) or Boehringer Mannheim Biochemicals (Indianapolis, Indiana).

The present invention is also directed to nucleic acid molecules produced by the methods of the invention, which may be cDNA molecules, especially full-length cDNA molecules, to vectors (particularly expression vectors) comprising these nucleic acid molecules and cDNA molecules and to host cells comprising these nucleic acid molecules, cDNA molecules, and/or vectors.

Recombinant vectors may be produced according to this aspect of the invention by inserting, using methods that are well-known in the art, one or more of the cDNA molecules or nucleic acid molecules prepared according to the present methods into one or more vectors. The vector used in this aspect of the invention may be, for example, a phage or a plasmid vector, and is preferably a plasmid. Preferred are vectors comprising *cis*-acting control regions to the nucleic acid encoding the polypeptide of interest. Appropriate *trans*-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker such as a tetracycline or ampicillin resistance gene for culturing in a bacterial host cell. Prior to insertion into such an expression vector, the cDNA or nucleic acid molecules of the invention should be operatively linked to an appropriate promoter.

Among vectors preferred for use in the present invention include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from Invitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2, pSV SPORT1, pCMVSPORT6 and pCMVSPORT available from Life Technologies, Inc. Other suitable vectors will be readily apparent to the skilled artisan.

The invention may be used in conjunction with any methods of cDNA synthesis that are well-known in the art (see, e.g., Gubler, U., and Hoffman, B.J., *Gene* 25:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol.* 152:316-325 (1987); Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989); PCT US98/19948; and WO 98/51699) to produce cDNA molecules or libraries. Other methods of cDNA synthesis which may advantageously use the present invention will be readily apparent to one of ordinary skill in the art.

Having obtained cDNA molecules or libraries according to the present methods, these cDNAs may be isolated for further analysis or manipulation. Detailed methodologies for purification of cDNAs are taught in the GENETRAPPER™ manual (Life Technologies), which is incorporated herein by reference in its entirety, although alternative standard techniques that are known in the art (see, e.g., Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989)) may also be used. The cDNA molecules or libraries produced by the invention may also be further manipulated by standard molecular biology techniques such as two hybrid analysis, cDNA normalization, sequencing and amplification. More particularly, the methods of the invention and the cDNA molecules or libraries produced by such methods may be used in combination with RT-PCR and 5' RACE technology (Life Technologies, Inc.) and differential display.

A variety of inhibitors and binding molecules are suitable for use in the present methods. Included among these inhibitors or binding molecules are

antibodies that bind to the above-described polypeptides having reverse transcriptase activity (such as anti-RT antibodies including anti-AMV RT antibodies, anti-M-MLV RT antibodies or anti-RSV RT antibodies) or to cap structure (e.g., anti-cap antibodies), and fragments thereof (such as Fab or F(ab')₂ fragments). Such antibodies may be polyclonal or monoclonal, and may be prepared in a variety of species according to methods that are well-known in the art. See, for instance, Sutcliffe, J.G., *et al.*, *Science* 219:660-666 (1983); Wilson *et al.*, *Cell* 37: 767 (1984); and Bittle, F.J., *et al.*, *J. Gen. Virol.* 66:2347-2354 (1985). Antibodies specific for any of the above-described reverse transcriptases or cap structures can be raised against the intact polymerase polypeptide or cap structures or one or more fragments thereof. These polypeptides or cap structures or fragments thereof may be presented together with a carrier protein (e.g., albumin) to an animal system (such as rabbit or mouse) or, if they are long enough (at least about 25 amino acids), without a carrier.

As used herein, the term "antibody" (Ab) may be used interchangeably with the terms "polyclonal antibody" or "monoclonal antibody" (mAb), except in specific contexts as described below. These terms, as used herein, are meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')₂ fragments) which are capable of specifically binding to a polypeptide having reverse transcriptase activity (such as a DNA polymerase or a reverse transcriptase) or cap structures or portions thereof.

The antibodies used in the methods of the present invention may be polyclonal or monoclonal, and may be prepared by any of a variety of methods (see, e.g., U.S. Patent No. 5,587,287). For example, polyclonal antibodies may be made by immunizing an animal with one or more polypeptides having reverse transcriptase activity or cap structures or portions thereof according to standard techniques (see, e.g., Harlow, E., and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (1988); Kaufman, P.B., *et al.*, In: *Handbook of Molecular and Cellular Methods in Biology and Medicine*, Boca Raton, Florida: CRC Press, pp. 468-469 (1995)). Alternatively, monoclonal antibodies (or fragments thereof) to be used in the

present methods may be prepared using hybridoma technology that is well-known in the art (Köhler *et al.*, *Nature* 256:495 (1975); Köhler *et al.*, *Eur. J. Immunol.* 6:511 (1976); Köhler *et al.*, *Eur. J. Immunol.* 6:292 (1976); Hammerling *et al.*, In: *Monoclonal Antibodies and T-Cell Hybridomas*, New York: Elsevier, pp. 563-681 (1981); Kaufman, P.B., *et al.*, In: *Handbook of Molecular and Cellular Methods in Biology and Medicine*, Boca Raton, Florida: CRC Press, pp. 444-467 (1995)).

It will be appreciated that Fab, F(ab')₂ and other fragments of the above described antibodies may be used in the methods described herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). Antibody fragments may also be produced through the application of recombinant DNA technology or through synthetic chemistry.

The invention also provides kits for use in accordance with the invention. Such kits comprise a carrier means, such as a box or carton, having in close confinement therein one or more container means, such as vials, tubes, bottles and the like, wherein the kit may comprise (in the same or separate containers) one or more host cells, one or more reverse transcriptases, one or more reverse transcription inhibitors, one or more cap binding molecules, one or more DNA polymerases, suitable buffers, one or more nucleotides and/or one or more primers (e.g., oligo(dT) for reverse transcription). The kits encompassed by this aspect of the present invention may further comprise additional reagents and compounds necessary for carrying out standard nucleic acid reverse transcription protocols.

It will be readily apparent to one of ordinary skill in the relevant art that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

Examples:

Example 1: Comparison of first strand cDNA synthesis with varying ratios of oligo (dT) primer/mRNA

This example compares first strand cDNA synthesis of the MAP4 gene with various ratios of oligo dT primer/starting mRNA. All components are available from Life Technologies, Inc., Rockville, Maryland, unless specified otherwise.

The master mix for Superscript II reverse transcriptase (SS II RT) was prepared as specified in Table 1 below.

Table 1

Component	μ l	μ l
5X SSII RT buffer	4	28
0.1 M DTT	2	14
10 mM dNTP	1	7
α - ³² P dCTP	0.5	3.5
Water	1.5	10.5
Total volume	9	63

The master mix for ThermoScript™ II RT (TS RT) (AMV RT α H- β H+) (see WO 98/47921) was prepared as specified in Table 2 below.

Table 2

Component	μ l	μ l
10X TS II buffer*	2	38
0.1 M DTT	2	38
10 mM dNTP	2	38
(α - ³² P dCTP	0.5	9.5
RNase OUT (40 u/ l)	1	19
Water	1.5	28.5
Total volume	9	171

*10X TS II buffer comprises 50 mM Tris-HCl (pH 8.4), 750 mM KCl, and 75 mM MgCl₂.

The master annealing mix was prepared by adding a 5 Kb MAP4 mRNA, oligo(dT)₂₅₋₃₀ and water to 5 tubes in the amounts specified in Table 3 below.

Table 3

Ratio of oligo (dT)/mRNA	1:1	2.5:1	5:1	10:1	50:1
Component	Volume	Volume	Volume (μl)	Volume	Volume
MAP4 mRNA (1 μg/μl)	5	5	5	5	5
Oligo(dT) ₂₅₋₃₀ (10 ng/μl)	2.6	6.5	13.1	-	-
Oligo(dT) ₂₅₋₃₀ (100 ng/μl)	-	-	-	2.6	13.1
Water	42.4	38.5	31.9	42.4	31.9
Total volume	50	50	50	50	50

The mixture was heated at 70°C for 10 minutes and then chilled on ice for 5 minutes.

Synthesis of first strand cDNA was done by adding 9 μl of the appropriate reverse transcriptase master mix, 10 μl of the master annealing mix and 1 μl of either SS II RT (200 units/ul) or TS II RT (15 units/ul) for a total volume of 20 μl as summarized in Table 4 below.

Table 4

Tube	Reverse Transcriptase	Temperature	Ratio of oligo (dT) ₂₅₋₃₀ / mRNA
1	SS II	45°C	1
2			2.5
3			5
4			10
5			50
1	TS II RT	45°C	1
2			2.5
3			5
4			10
5			50

6	TS II RT	50°C	1
7			2.5
8			5
9			10
10			50
11	TS II RT	55°C	1
12			2.5
13			5
14			10
15			50

The reactions were incubated for 1 hour at 45°C for SS II RT and at 45, 50 or 55°C for TS II RT. The tubes were placed on ice to complete the reaction. 18 µl first strand cDNA of the reaction tube was precipitated and re-suspended in 10 µl of water. 5 µl of the first strand cDNA was mixed with 5 µl of standard loading buffer (60 mM NaOH, 4 mM EDTA, 0.1% bromophenol blue), and loaded onto 1.4% alkaline agarose gel for analysis. * * *

* * * *

The results show that by reducing the molar ratio of oligo(dT) primer/mRNA (preferably to 1:1) internal priming with reverse transcriptase was almost entirely eliminated.

Example 2: Comparison of first strand cDNA synthesis under standard and hot start conditions

This experiment was designed to compare first strand cDNA synthesis of the MAP4 gene with standard reaction and hot start conditions.

The annealing mix was prepared by mixing 1 µg of MAP4 mRNA and biotinylated *Not* I oligo(dT)₂₅ primer ((Biotin)₄ GACTAGTTCTAGAT CGCGAGCGG CCGCCCTTTT TTTTTTTTTTTT TTTTTTTT; see WO 98/51699) in the desired molar ratio of oligo (dT)/mRNA of 0:1, 1:1 or 15:1 in thin-walled PCR tubes and bringing the volume up to 10 µl with water. If

several tubes are identical, they may be made in one batch and aliquotted accordingly. The annealing mix was kept on ice.

The master mix for Superscript II reverse transcriptase (SS II RT) was prepared as specified in Table 5 below.

Table 5

Component	μl	μl
5X SSII RT buffer	4	28
0.1 M DTT	2	14
10 mM dNTP	1	7
α - ³² P_dCTP	0.5	3.5
SSII RT (200 u/ μl)	1	7
Water	1.5	10.5
Total volume	10	70

The SS II RT master mix was then divided into two equal aliquots, one for processing with standard reaction temperatures (batch 1) and one for processing with hot start reaction temperatures (batch 2). To allow for condensation, an additional 10% volume of water was added to batch 2. All mixes were kept on ice.

Synthesis of first strand cDNA was begun by briefly spinning tubes containing annealing mix to collect droplets, placing the tubes in a thermocycler and then heating them to 70°C for 10 minutes. After this 10 minute cycle at 70°C, the tubes of annealing mix for batch 1 were immediately removed to ice. The tubes of annealing mix for batch 2 were allowed to cool to 45°C in the thermocycler while the batch 2 master mix was placed in the thermocycler and incubated at 45°C for 5 minutes. After the 5 minute incubation, 11 μl of the master mix for batch 2 was added to each batch 2 annealing tube and mixed with a pipette 2 times. Care was taken not to spin the tubes to avoid lowering the temperature.

10 μl of the master mix for batch 1 was added to each batch 1 annealing tube. The batch 1 tubes were lightly vortexed and briefly centrifuged to collect condensation droplets. The batch 1 tubes were then returned to the thermocycler and the tubes from both batch 1 and 2 were incubated at 45°C for one hour.

5 µl of the first strand cDNA from each tube was mixed with 5 µl of standard loading buffer (60 mM NaOH, 4 mM EDTA, 0.1% bromophenol blue) and loaded onto 1.4% alkaline agarose gel for analysis. * * *

* * * *

First strand cDNA was also synthesized with TS II RT using 15 units of TS II RT per µg mRNA using a biotinylated oligo(dT)/mRNA ratio of 1:1 and 15:1. The same protocol described above was followed, except that the temperature was varied to 50°C.

* * * *

The results indicated that by dropping the reaction temperature to the reverse transcriptase reaction temperature after denaturation of the primer and mRNA mixture, the reaction was started directly and internal priming was avoided entirely.

Example 3: Synthesis of double strand cDNA by controlling the reaction temperature and the concentration of salt and RNase

This example describes the synthesis of double stranded cDNA by controlling the reaction temperature and the concentration of salt and different ribonuclease (RNases) during the treatment of the cDNA/mRNA hybrids after first strand cDNA synthesis.

First strand cDNA was synthesized as described above in Example 2 and digested with either RNase I or RNase A as further described below.

RNase I digestion of first strand cDNA was done by re-suspending the first strand cDNA in 180 µl of water and 20 µl of 10X RNase I buffer (100 mM Tris-HCl (pH 7.5), 50 mM EDTA, 2 M sodium acetate). 2.5 units of RNase I (1 unit/µg mRNA) were added and the mixture was mixed well. The RNase I digestion mixture was incubated at 25°C for 30 minutes and extracted with phenol/chloroform once. The supernatant was precipitated with 1 R1 of glycogen, 100 µl of ammonium acetate and 800 µl of ethanol.

RNase A digestion of first strand cDNA was done by re-suspending the first strand cDNA in 200 µl of digestion buffer (10 mM Tris-HCl (pH 7.5), 250 mM NaCl). 12.5 ng of RNase A (5

ng/ μ g mRNA) were added and the mixture was mixed well. The RNase A digestion mixture was incubated at 25°C for 30 minutes and extracted with phenol/chloroform once. The supernatant was precipitated with 1 μ l of glycogen, 100 μ l of ammonium acetate and 800 μ l of ethanol.

Example 4: Enrichment of the full-length cDNA clones with cap-binding proteins

This example describes enrichment of full-length cDNA clones with the capbinding protein eIF4E.

cDNA was prepared by precipitating the RNase I treated first strand cDNA described in Example 3 above and washing with 70% ethanol. The resulting pellet was dried at room temperature for 5 minutes, and re-suspended in 210 μ l of 10 mM KPO₄, 100 mM KCl, 2 mM EDTA, 6 mM DTT and 5% glycerol. The cDNA was stored on ice.

eIF4E glutathione sepharose 4B beads were prepared by first mixing glutathione sepharose 4B beads (Pharmacia, Sweden) well. To prepare eIF4E beads, a recombinant host cell expressing GST tagged eIF4E protein (the eIF4E gene was cloned into a GST fusion vector to create a N-terminal GST-eIF4E fusion gene) was grown and the fusion protein was purified by standard techniques. Thus, the invention also relates to recombinant host cells expressing eIF4E protein (particularly as fusion proteins), to vectors comprising the genes expressing such proteins or fusion proteins and to the recombinant proteins or fusion proteins produced. In the present invention any tag can be used (e.g., His Tag, GST tag, HA tag, *Trx* tag, etc.). Such tags may be positioned at the carboxy and/or N-terminal region of the eIF4E gene.

The GST-eIF4E fusion protein was complexed with sepharose 4B beads by glutathione coupling using glutathione sepharose 4B beads (Pharmacia Biotech) following the manufacturers protocols. 200 μ l of the beads were transferred to a 1.5 ml microcentrifuge tube, centrifuged for 1 second, and 75 μ l of supernatant was removed. The beads were washed twice with 1 ml of reaction buffer (10 mM KPO₄, 100 mM KCl, 2 mM EDTA, 6mM DTT and 5% glycerol), and re-suspended in 258 μ l of reaction buffer, followed by the addition of 42 μ l (18 pmoles/ μ l) of eIF4E protein (600 pmoles/10 μ l beads). The mixture was mixed on a head to head roller at 4°C for 30

minutes. The mixture was then centrifuged for 1 second, and the supernatant was removed. The beads were washed twice with 1 ml of reaction buffer and once with 1 ml of 25 µg/ml yeast tRNA in reaction buffer. 20 µl of reaction buffer and 5 µg of yeast tRNA were then added to the beads. 200 µl of RNase I treated cDNA was added to the beads, and the content was mixed on a roller at room temperature for 1 hour. After 1 hour, the mixture was centrifuged for 1 second, and the supernatant was removed. The beads were washed twice with 1 ml of reaction buffer and once with 1 ml of 500 µM GDP in reaction buffer. The cDNA was eluted twice with 250 µl of 500 µM GDP in reaction buffer. The eluted solutions were pooled and centrifuged for 1 minute to remove the beads. The eluted cDNA was extracted twice with an equal volume of phenol/chloroform. The cDNA was divided into two tubes and precipitated with 1 µl of glycogen, 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of ethanol.

Example 5: Evaluation of the cDNA library

To evaluate the quality of the cDNA libraries constructed with the above-described full-length methods, the MAP4 gene (5-6 kb) and other genes was selected as the target genes. MAP4 and other cDNA clones were isolated from libraries constructed by standard methods well-known in the art (see SuperScript™ Plasmid Manual, Life Technologies, Inc.) and the above-described full-length methods with 3' and 5' GeneTrapper cDNA Positive Selection System (Life Technologies, Inc., Rockville, Maryland). The positive clones were size analyzed by PCR. Tables 6 and 7 below summarizes the results of the enrichment of full-length cDNA clones in human fibroblast cDNA libraries constructed with methods well-known in the art (control) and the full-length methods described above (full-length method).

Table 6

Gene	% full-length with 5' GeneTrapper	% full-length with 3' GeneTrapper
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	control*	full-length method	control*	full-length method
MAP4 (5-6 kb)	12.8	90.3	6.25	37.5

*The control library was constructed with SS II RT using known methods

Table 7

Gene name	Full-length of gene (Kb)	% Full-length by 5' GeneTrapper
MAP4 (Microtubule-associated protein_4)	5/6	90.3
β -Adaptin*	3.8/5.7	90.0
TR (Transferrin receptor)	5.0	45.0
PTK (Protein tyrosine Kinase)	3.0	84.4
RPA (DNA Replication protein A)	1.4	98.0

* There are two members of the genes, 3.8 kb and 5.7 kb in the family.

These results show that the full-length methods described above yielded >90% full-length cDNA clones with the 5' GeneTrapper system, compared to <13% using standard methods. Furthermore, the above-described- full-length methods yielded >37% full-length clones with the 3' GeneTrapper system, as compared to <7% using standard methods.

Example 6: First strand cDNA synthesis, RNase I digestion and eIF-4E capture

All conditions and parameters described above in Examples 2, 3 (RNase I) and 4 were followed, except for the following: 4 reactions of 10 μ g of human fibroblast cytoplasmic mRNA were used per reaction (see WO 98/45311); the biotinylated

primer-adapter (Biotin)₄-
GACTAGTTCTAGATCGCGAGCGGCCGCCC(T)₂₅ was used at a 1:1 primer/mRNA molar ratio; TS II RT was used at 50°C; and SS II RT was used at 45°C. Table 8 below summarizes the first strand cDNA and eIF-4E capture results.

Example 7: Second strand cDNA synthesis

Second strand cDNA was synthesized by first dissolving each of the four reaction pellets obtained in Example 6 above in 104 µl of DEPC-treated water and then adding the following reagents to each reaction:

4 µl of 5X First Strand Buffer*

30 µl of 5X Second Strand Buffer*

2 µl of 0.1 M DTT

4 µl of 10 mM dNTPs

1 µl of *E. coli* DNA ligase (10 units/µl)

1 µl of *E. coli* RNase H (2 units/µl)

4 µl of *E. coli* DNA polymerase (10 units/µl)

- see SuperScript Plasmid System manual (Life Technologies, Inc., Rockville, Maryland)

These reactions mixtures were then incubated for 2 hours at 16°C. 2 µl of T4 DNA polymerase (5 units/µl) was added and incubation at 16°C was continued for 5 more minutes.

Example 8: Streptavidin Bead Preparation

During the last 30 minutes of the 2 hour second strand reaction described in Example 7 above, streptavidin paramagnetic beads were prepared as follows. Streptavidin paramagnetic beads (Seradyn) were gently mixed by pipetting until the beads were completely re-suspended. 150 μ l of the mixed beads were transferred to the bottom of a microcentrifuge tube for each reaction. The tubes were inserted into a Magna-Sep Magnetic Particle Separator (Life Technologies, Inc., Rockville, Maryland) (the magnet) and let sit for 2 minutes. While the tubes were in the magnet, the supernatant was removed by pipetting and 100 μ l of TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) was immediately added to the beads.

The tubes were then removed from the magnet and the beads were gently re-suspended by finger tapping or vortexing at the lowest setting. The tubes were reinserted into the magnet. After 2 minutes, the supernatant was removed, the beads were re-suspended in 160 μ l of binding buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 M NaCl) and the tubes were placed into a microcentrifuge tube rack.

Example 9: Capture of the double-stranded cDNA library

After incubating the second strand reaction with T4 DNA polymerase as described in Example 7 above, the reaction mixtures were placed on ice and 10 μ l of 0.5 M EDTA was added. Then the cDNA library was captured according to the following procedure (see generally WO 98/51699).

The paramagnetic beads prepared according to Example 8 were transferred to the second strand reaction mixture tubes and gently mixed by pipetting and the suspension was incubated for 60 minutes at room temperature. The tubes were then inserted into the magnet. After 2 minutes, the supernatant was removed and discarded.

100 μ l of wash buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 500 mM NaCl) was added to the beads, the beads were re-suspended by finger tapping or gently vortexing at the lowest setting and the tubes were re-inserted into the magnet for 2 minutes. The supernatant was removed and discarded. This washing step was repeated

one more time and then 100 µl of wash buffer was added to the beads. The tubes were then again inserted into the magnet for 5 minutes.

Example 10: *Not* I Digestion

After the 5 minute incubation described in the last step of Example 9, the supernatant was removed and discarded from the paramagnetic beads and 41 µl of autoclaved, distilled water, 5 µl of REact 3 buffer, 4 µl of *Not* I was added and the beads were mixed well by pipetting. The reaction was then incubated for 2 hours at 37°C. The tubes were then inserted into the magnet for 2 minutes and the supernatant containing the cDNA library was transferred to fresh tubes.

50 µl of phenol: chloroform:isoamyl alcohol (25:24:1) was added to the supernatant, the solution was vortexed thoroughly, and then centrifuged at room temperature for 5 minutes at 14,000 x g. 45 µl of the upper, aqueous layer was carefully removed and transferred to fresh microcentrifuge tubes. 23 µl of 7.5 M ammonium acetate, 1 µl of glycogen (20 µg) and 172 µl of ethanol (-20°C) was added. The solution was mixed well and stored on dry ice (or -70°C freezer) for 15 min.

The ethanol solution was then centrifuged at 4°C for 30 minutes at 14,000 x g. The supernatant was carefully removed from the small pellets. 100 µl of 70% ethanol was added and the tubes were centrifuged at room temperature for 2 minutes at 14,000 x g. The ethanol was removed and the pellets were dried in a speed-vac for 2 minutes or until dry. The pellets were then dissolved in 20 µl of TE buffer (10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA). The final yield of cDNA was determined by the Cerenkov counts (see Table 8 below).

Table 8

Reverse Transcriptase	standard (S) or varied (V) <u>temperature</u>	% Incorporation (ng of eDNA)	Amount of cDNA after eIF-4E <u>capture</u>
TS II RT	S	27% (2,720 ng)	512 ng

TS II RT	V (hot start)	26% (2,640 ng)	473 ng
SS II RT	S	46% (4,560 ng)	306 ng
SS II RT	V (hot start)	47% (4,730 ng)	363 ng

Example 11: Ligation of cDNA to the vector and introduction into E coli

From 10 to 30 ng of the un-fractionated or size fractionated (≥ 1.5 kb by low melting gel electrophoresis) cDNA was ligated into a vector pCMVSPORT 6 (Life Technologies, Inc.). This ligation was introduced into *E. coli* by electroporation as described in the SuperScript Plasmid System manual (Life Technologies, Inc., Rockville, Maryland), except that the cloning vector was pre-digested with *Not* I and *Eco* RV.

Sequence analysis of randomly selected clones from the cDNA library constructed (304 clones) were analyzed by 5' and 3' sequencing to determine the total percentage of full-length random clones in the cDNA library. Sequences were compared for homology with GeneBank sequences. The results are summarized in Table 9 below. Based on the results, approximately 68% of the random clones were full-length (including known full-length clones and unknown full-length clones). Thus, approximately 17% unknown full-length clones were obtained from the human fibroblast cytoplasmic mRNA library.

Table 9

	Number of Clones	Percentage
Total Sequences	304	73.3%
Sequences with Homology	223	51%
Full-Length_Cloness	114	17%
Potentially Full-Length	39	17%
Partial Clones	70	31%

Example 12: RNase Assay

First strand cDNA was treated with RNase A at 1000 ng/ μ g mRNA in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) and RNase 125 to 40 u/ μ g mRNA in TEN (10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 200 mM Sodium Acetate) at $\sim 37^{\circ}\text{C}$ essentially as

described in Example 3. However, this treatment with large amounts of RNase at elevated temperatures resulted in libraries containing very small average cDNA insert size (about 200 bp). Therefore, a second strand cDNA assay was developed to determine the optimal amount of RNase needed.

First strand cDNA (radioactively labeled and non-radioactively labeled) was synthesized using HeLa mRNA at 500 ng of RNA/reaction. The first strand cDNA was precipitated with ethanol and dissolved in DEPC-treated water. The cold first strand cDNA was added to RNase buffer with different amounts of RNase. After incubation for 30 minutes at 25°C, the treated cDNA was extracted with phenol:chloroform and precipitated with ethanol. The treated cDNA was dissolved in DEPC-treated water, a second strand cDNA reaction was performed with ³²PdCTP plus and minus RNase H. The reaction was extracted with phenol:chloroform and precipitated with ethanol. Equal amounts of cpm was electrophoresed into a 1.4% alkaline-agarose gel.

* * * *

These gel analysis demonstrated that a concentration of 1.25 ng of RNase A * * * or 0.5 units of RNase I * * * may be optimal to use with 500 ng of starting mRNA.

Example 13: Preparation of antibodies against cap structure

The antibody to cap was generated using m⁷guanosine-KLH as the antigen. 1200 hybridomas were plated and only 120 colonies were generated. Of these only 6 colonies were positive for cap. After further analysis, 3 were determined to have the affinity required. The first screen ELISA consists of binding m⁷guanosine-BSA to an ELISA plate, block with BSA, bind hybridoma supernatants, react with secondary antibody and determine positives via a colorimetric reaction with BCIP/NPT. The secondary screen included incubating appropriate dilutions of the hybridoma supernatants with either 0.1 mM m⁷GTP, 0.1 mM cap analog M⁷G⁵ppp⁵G, 0.5 mM m⁷guanosine or 0.5 mM GTP. The pretreated supernatant was then used in the standard ELISA